STRUCTURAL SPECIFICITY OF FLAVONOIDS TO SELECTIVELY INHIBIT STARCH DIGESTIVE ENZYMES FOR TRIGGERING THE GUT-BRAIN AXIS

by

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To my family

Thank you for your non-stop support,

I am so lucky to have you all!

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ABSTRACT

In this study, structural specificity of flavonoids was investigated to selectively inhibit starch digestive enzymes to stimulate the ileal-brake by triggering glucagon-like peptide-1 (GLP-1) through distal small intestine starch digestion which can regulate food intake and appetite. The double bond between C2 and C3 on flavonoid's chemical structure plays a critical role to inhibit human pancreatic α-amylase, leading to π-staking interaction. Meanwhile, the hydroxyl group at C3 on the backbone benzopyran ring is intimately related to inhibition of the mucosal α-glucosidases. This selective inhibition is likely the result of fundamental differences in the protein structures of α-amylase and α-glucosidases, as they belong to different glycosyl hydrolase Families 13 and 31 (GH13 and GH31). α-Amylase has the catalytic active sites located in wide and shallow grooves on the protein structure, while α-glucosidases possess the narrow and deep catalytic pocket. In an acute study done on mice, luteolin, which had the higher degree of selectivity toward α-amylase, showed a slow and sustained postprandial glycemic response with a reduced blood glucose peak and extended high glucose profile, compared to 3',4'-dihydroxylflavonol as the selective α-glucosidases specific inhibitor. Quercetin was inhibitory of both α-amylase and α-glucosidases. Glycemic profiles in mice confirmed in vitro analysis of the inhibitory selectivity of the flavonoids tested. Additionally, the extended glycemic response with luteolin was accompanied the higher secretion of GLP-1 at extended postprandial times by delivering more starch portion into the distal small intestine where the ileal-brake and gut-brain axis activation takes place. Overall, selective inhibition of α-amylase by flavonoids potentially could be considered as a key approach to control glucose release from starch with slow and extended, but still complete, digestion for improved glycemic response and minimized adverse side effects that result from severely restricting or even shutting down starch digestion by pharmaceutical grade inhibitors.

CHAPTER 1. INTRODUCTION

It is estimated that over 400 million people suffer from diabetes due to pancreatic β-cell dysfunction or insulin resistance with impaired glucose tolerance. It is generally considered that management of the postprandial glucose level is critical for prediabetic or diabetic patients. Pharmaceutical agents such as acarbose, miglitol, and voglibose have been used to treat or prevent carbohydrate diet-related metabolic diseases by controlling starch digestion rate with a strong inhibition property for carbohydrate digestive enzymes. Unfortunately, they often cause severe gastrointestinal side effects such as diarrhea, bloating, and flatulence, that result from the dumping of the undigested starch fractions into the colon. An alternative approach is to selectively inhibit starch digestive enzymes to regulate glucogenesis with slow, but complete, starch digestion.
Flavonoids have been shown to inhibit starch digestive enzymes as natural inhibitors with partial inhibition property (Forester, Gu, & Lambert, 2012; Williamson, 2013). Depending on the chemical ring structure of flavonoids, they have different inhibition activities against α-amylase and α-glucosidases due to the different protein structure of starch digestive enzymes (Bernardi, 2015; Williams et al., 2015). Therefore, many studies have been conducted to understand the structural requirement of flavonoids to inhibit starch digestive enzymes (Piparo, Scheib, Frei, Williamson, Grigorov, & Chou, 2008; Xiao, Kai, Yamamoto, & Chen, 2013; Xiao, Ni, Kai, & Chen, 2013).

Control of starch digestion rate by inhibiting enzyme activity could have multiple positive health implications, including improvement of postprandial glycemic response with slow and sustainable blood glucose level and stimulation of the gut-brain axis, through distal digestion in the small intestine, by triggering GLP-1 to regulate food intake and appetite.

This study has the three different objectives. First is to develop a new technical method to evaluate the inhibition property of flavonoids against starch digestive enzymes. Even though there are the conventional colorimetric methods such as DNS and GOPOD to measure the digested products, flavonoids can be involved in the chemical reactions due to antioxidant activity and inhibition property of working enzymes, thus acting as a confounding factor. There is a need to develop a precise and accurate method to measure the inhibition property of natural phenolic inhibitors. Second is to understand the structural requirement of flavonoids to find inhibitors with

strong effect on α-amylase and the α-glucosidases. α-Amylase has the catalytic active site located in a wide and shallow groove on protein surface, while α-glucosidases have a narrow and deep pocket at the catalytic site. Based on differences in protein structure, different flavonoids will show different inhibition activities depending on their chemical structures. Therefore, a screening of flavonoid structures will give insight to find the optimal ring structure to inhibit starch digestive enzymes. Finally, there is a need to know which type of enzyme inhibition with flavonoids, selected to have strong effect from the screening study, are more contributory to control starch digestion rate, modulate postprandial glycemic response, and if possible activate enteroendocrine L-cells. The thesis purpose is to find efficacious flavonoids and understand selective inhibition of starch digestive enzymes to slow digestion of starch and glucose release, but with more complete digestion than can be provided with pharmaceutical-grade inhibitors. The goal is to reduce digestion rate to improve glycemic response and prevent gastrointestinal side effects. Overall, knowledge obtained from this study will provide insights into future development of functional food and antidiabetic agents for controlling starch digestion rate.

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CHAPTER 2. LITERATURE REVIEW

2.1 Introduction

Starch is a homogeneous glucose polymer consisting of linear amylose and branched amylopectin (Badenhuizen, 1963) , and it provides a considerable portion of energy in the human nutrition (BeMiller, 2019). Starch is digested to glucose in the gastrointestinal tract for absorption as an energy source by amylolytic α-amylases from the salivary gland and pancreas, and brush border glucogenic α-glucosidases in the small intestine. Glucose digested from starch by the starch degrading enzymes is further absorbed into the blood stream via glucose transporters located at the luminal surface of the enterocytes (Stipanuk, 2006). Starch is nutritionally classified into three different categories based on their digestibility in the small intestine: rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) (Englyst, Kingman, & Cummings, 1992). RDS leads to a sharp increase in postprandial blood glucose levels, and chronic consumption of starch contained high amounts of RDS may initiate hyperglycemia, hyperinsulinemia, and insulin resistance. Thus, modulation of starch digestion rate has been a focus to reduce the risk factors of diet-related metabolic diseases such as obesity and type 2 diabetes (Zhang & Hamaker, 2009).

Starch digestion rate can be controlled by inhibiting hydrolytic activities of α-amylases and α-glucosidases using the pharmaceutical agents (e.g., acarbose, miglitol, and voglibose) (Chiasson et al., 2003; Kootte et al., 2012). Unfortunately, the use of these drugs often causes side effects, such as abdominal pain, diarrhea, and flatulence, due to dumping of a large portion of undigested starch into the colon (Chiasson, Josse, Gomis, Hanefeld, Karasik, & Laakso, 2002; Jean Louis Chiasson, 1994). The considerable restriction of starch degradation is derived from poor specificity and strong activity of the inhibitors toward the enzymes, resulting in substantial inhibition or even shutting down of all enzyme activities (Bernardi, 2015; Williams et al., 2015). An alternative approach is to use certain compounds with higher specificity and lower activity for the individual enzymes to promote selective and partial inhibition of the starch digestive enzymes. This inhibition could modulate glucogenesis with slow, but still complete, digestion of starch in the small intestine for improved glycemic response and minimized adverse side effects (Lim, Kim, Shin, Hamaker, & Lee, 2019; Simsek, Quezada-Calvillo, Ferruzzi, Nichols, & Hamaker, 2015).

Considerable attention has been given towards the use of dietary polyphenols as natural inhibitors for the starch digestive enzymes to modulate starch digestion rate within the gastrointestinal tract (Forester, Gu, & Lambert, 2012; Nyambe-Silavwe & Williamson, 2016). Some polyphenols showed selective inhibition property against α-amylases and α-glucosidases. Tarling *et al*. (2008) discovered a specific inhibitor of human pancreatic α-amylase, which was extracted from *Crocosmia crocosmiiflora*. The inhibitor has a high level of selectivity for α-amylase with low or even no inhibition of α-glucosidases. It was also noted that luteolin differently inhibited porcine pancreatic α-amylase and α-glucosidases from rat intestinal powder, showing different inhibition constants (K_i) of 0.22 and 2.63 mM, respectively (Lim, Zhang, Ferruzzi, & Hamaker, 2019). Additionally, Kim *et al*. (2000) reported that the tested flavonoids (amentoflavone, genistin, hyperin, and inkgetin) showed different inhibition of porcine pancreatic α-amylase and yeast α-glucosidases.

The selective inhibition of starch digestive enzymes by polyphenols is likely the result of different protein structures of α-amylase and α-glucosidases that belong to glycosyl hydrolase families 13 and 31 (GH13 and GH31), which results in different binding interactions with polyphenols (Lim, Zhang, et al., 2019; Williams et al., 2015). Salivary and pancreatic α-amylases have catalytic active sites located in a wide and shallow groove on the protein surface, behaving in an endo-acting manner, whereas brush border α-glucosidases in the small intestine have a narrow and deep catalytic pockets with an exo-acting mechanism (Davies & Henrissat, 1995; Henrissat & Davies, 1997). Therefore, comprehension of the interaction of dietary polyphenols and starch digestive enzymes could further contribute to understanding the protein-ligand interaction, and to find certain structures of polyphenols which have a higher selectivity toward α-amylases and α-glucosidases for selectively inhibiting enzyme activity.

The purpose of this chapter is to review protein structure of α-amylases and α-glucosidases, understand how the starch enzymes work in a complementary manner to cleave starch into glucose, investigate binding interaction of the starch enzymes with polyphenols, and give insight into potential directions for future research.

2.2 Characteristics of starch digestive enzymes

2.2.1 Classification in Carbohydrate-Active enZymes system (CAZy)

Starch digestive enzymes, α-amylase and α-glucosidase, are all members of glycosyl hydrolase (GH) in Carbohydrate-Active enZymes system (CAZy) (Cantarel, Coutinho, Rancurel, Bernard, Lombard, & Henrissat, 2009), and α-amylase and α-glucosidase can be further classified into different subgroups based upon their distinct amino acid sequences (Table 1). α-Amylase from mammals, including humans, is considered to be an intensively studied amylolytic enzyme in clan-H, family 13, and subfamily 24 within the GH groups (Janecek, Svensson, & Macgregor, 2014). α-Glucosidases from humans belong to clan-D, family 31, and subfamily 1 among the GH families, accounting for the vast majority of the characterized enzymes in GH31 (Ernst, Lo Leggio, Willemoës, Leonard, Blum, & Larsen, 2006; Naumoff, 2011). GHs can hydrolyze glycosidic bond via two major mechanisms which result in a product with inversed or retained anomeric configuration. Both α-amylase and α-glucosidase act by the retaining mechanism, leading to the retention of the stereochemistry at the anomeric center (McCarter & Stephen Withers, 1994; Vuong & Wilson, 2010). In addition, GH13 and GH31 contain the $(\beta/\alpha)_8$-barrel catalytic domain fold and share aspartate (Asp) as the catalytic residue at active site (Janeček, Svensson, & Macgregor, 2007).

2.2.2 Protein structures of starch digestive enzymes

α-Amylases from the salivary gland and pancreas consist of 496 amino acids in a single polypeptide chain with a molecular weight of 56 kDa (Nakamura et al., 1984) and share a higher degree of primary structure homology with 97% identical in amino acid sequence (Brayer, Luo, & Withers, 1995). Salivary and pancreatic α-amylases have only 14 amino acid substitutions in the vicinity of the active site (Ramasubbu, Paloth, Luo, Brayer, & Levine, 1996), causing somewhat different action modes and substrate specificities (Kuroda, 1988). Human α-amylases have five glucosyl unit binding subsites with the catalytic site positioned between the third and fourth subsites from the non-reducing end, thus hydrolyzing starch mainly to maltose and maltotriose (Brayer et al., 2000; Robyt & French, 1970; Seigner, Prodanov, & Marchismouren, 1987). Furthermore, the catalytic active sites of salivary and pancreatic α-amylases are located in an open

cleft at the enzyme surface. The open cleft allows a random binding of several glucosyl units within starch chain (Bernardi, 2015; Davies et al., 1995; Henrissat et al., 1997).

The mucosal α-glucosidases in the small intestine are composed of two individual protein complexes which are termed maltase-glucoamylase (MGAM) and sucrase-isomaltase (SI) (Figure 1). Each protein complex has two active subunits which are located at the luminal C-terminal domain and membrane-proximal N-terminal domain of the original protein (Sim, Quezada-Calvillo, Sterchi, Nichois, & Rose, 2008). The N-terminal subunits of MGAM and SI are anchored onto the brush border membrane via an *O*-glycosylated linker in the small intestine (Jones et al., 2011). Each subunit of α-glucosidases contains about 900 amino acids and all four individual subunits share 40-60% homology of amino acid sequence with a range of molecular weight from 120 to 140 kDa. N-terminal subunits of the α-glucosidases (Nt-MGAM and Nt-SI) possess two sugar binding subsites with cleavage taking place at the catalytic site between the first and second subsites from the non-reducing end, while C-terminal glucosidases (Ct-MGAM and Ct-SI) have four substrate binding sites, making it more suitable to cleave longer oligosaccharide chains compared to Nt-MGAM and Nt-SI (Davies, Wilson, & Henrissat, 1997; Ren et al., 2011). In addition, brush border α-glucosidases in the small intestine have the catalytic active site in the shape of a small and deep pocket to nip off the terminal unit of oligosaccharide (Bernardi, 2015; Davies et al., 1995; Henrissat et al., 1997).

2.3 Control of starch digestion rate

2.3.1 Starch digestion process

Starch must be converted to free glucose in the small intestine to utilize it as an energy source (Edwards et al., 2015). In humans, starch is digested by six different digestive enzymes (α-amylases from salivary gland and pancreas, and four different subunits of the mucosal α-glucosidases in the small intestine) in the gastrointestinal tract (Lin, Hamaker, & Nichols, 2012; Lin, Lee, et al., 2012). The starch component in most foods can be first broken down to linear and branched maltooligosaccharides (mainly maltose, maltotriose, and α-limit dextrins) by the salivary and pancreatic α-amylases (Lee et al., 2012). Then, the α-amylolysis products are further digested

to glucose by the mucosal α-glucosidases in the small intestine, which is absorbed and circulated into the blood stream (Quezada-Calvillo et al., 2007; Quezada-Calvillo et al., 2008).

All subunits of the musical α-glucosidases have exo-hydrolysis activity on α-1, 4 glycosidic linkages and belong to the GH 31 family of glycohydrolases (Semenza, Auricchio, & Rubino, 1965). Each MGAM and SI subunit has different catalytic properties. In MGAM, Ct-MGAM has higher capacity to digest the longer maltooligosaccharides, while Nt-MGAM hydrolyzes predominantly maltose. Furthermore, Ct-SI is involved in the hydrolytic activity of the α-1, 2 linkage of sucrose, and Nt-SI cleaves the α-1, 6 linkage of isomaltose and branched α-limit dextrins (Lin, Lee, et al., 2012; Lin, Nichols, et al., 2012).

2.3.2 Implication of modulation of starch digestion rate

Type 2 diabetes is one of the most common chronic diet-related metabolic diseases in the world. The number of individuals with type 2 diabetes has risen from 108 million in 1980 to 422 million in 2014. On this basis, the prevalence of type 2 diabetes is expected to nearly double by 2030 (Rathmann & Giani, 2004). Type 2 diabetes is a chronic disease from a lack of insulin stimulation of target muscle and adipose cells due to insulin resistance (Vijan, 2010). Type 2 diabetes can damage many major organs, including the heart, blood vessels, eyes, kidneys, and nerves (Temelkova-Kurktschiev et al., 2000). The fundamental causes of type 2 diabetes include lifestyle, genetic, environmental factors, and eating behavior, related to energy imbalance between calories consumed and expended (Ripsin, Kang, & Urban, 2009). In recent years, epidemiological studies suggested that low glycemic index (GI) carbohydrates can be considered as an approach to manage or treat type 2 diabetes by reducing the postprandial blood glucose level (Jenkins et al., 2002; Roberts, 2000). The concept of GI, first introduced by Jenkins (Jenkins et al., 1981), can be described as the area under the blood glucose response curve in 120 min after food intake, compared to control foods (glucose or white bread). GI foods can be mainly categorized into two groups: low GI foods (≤ 55), and high GI foods (≥ 70) (Atkinson, Foster-Powell, & Brand-Miller, 2008). Low GI foods result in slower glucose release into the bloodstream, while high GI foods cause rapid and sudden increase of the blood glucose level (Brand-Miller, 2007). Thus, low GI diets are associated with reduced risk of diet-related metabolic disorders (Ludwig, 2002). Starch is classified into three nutritional types based on the *in vitro* Englyst assay. RDS is the digested

portion within the initial 20 min digestion and RDS is strongly related to high GI response profile. RDS can be quickly utilized as an energy source in the proximal part of the small intestine and causes a rapid increase of the blood glucose level. The sharp glucose peak can affect the maintenance of blood glucose homeostasis. On the other hand, SDS is the digested fraction between 20 and 120 min and has slower digestion rate than RDS. SDS can be applied to obtain the decreased glucose spike and prolong the postprandial blood glucose level. While both RDS and SDS can be digested, RS cannot be digested by starch digestive enzymes in the gastrointestinal tract. RS enters the colon and then it can be fermented by the gut microbiota as dietary fiber (Lehmann & Robin, 2007). Chronic consumption of high GI foods, which lead to large perturbations in blood glucose levels, may initiate hyperglycemia, hyperinsulinemia, and insulin resistance (Wolever, 2003). Thus, low GI foods are believed to control postprandial blood glucose level for reducing the risk of chronic diet-related metabolic disorders.

A concept of an extended glycemic index (EGI) was proposed to specify extended glucose release over a prolonged time period (G. Zhang & Hamaker, 2009). EGI could be described as low GI with extended and moderated blood glucose level over the entire glycemic response profile. The extended glucose release can induce glucose to enter the distal part of the small intestine and then promote the ileal brake by stimulating the secretion of glucagon-like peptide 1 (GLP-1) and peptide YY (PYY), which have a function to regulate appetite and food intake (Jenkins et al., 2002). Thus, it is expected that the secretion of GLP-1, and perhaps the gut-brain axis, may be applied to manage body weight (de Graaf, Blom, Smeets, Stafleu, & Hendriks, 2004).

2.4 Dietary flavonoids

Polyphenols are present in fruits, vegetables, and grains. The most common of polyphenols in the diet are flavonoids. Dietary flavonoids are classified into five major groups, including flavonol, flavone, flavanol, flavanone, and isoflavanone. Their structures are represented by two phenyl rings with a heterocyclic ring. Flavonoids have a C6-C3-C6 backbone in which the two C6 units are of phenolic nature (Williams et al., 2015).

2.5 Utilization of flavonoids as natural inhibitors toward starch digestive enzymes

Dietary phenolic compounds have received significant attention due to their potential for modulating postprandial blood glucose level by inhibiting starch digestive enzymes (Williamson, 2013). Although there are well known strong natural and synthetic inhibitors against starch digestive enzymes, such as acarbose, miglitol and voglibose, the use of these inhibitors often results in severe gastrointestinal side effects such as abdominal pain, bloating, and diarrhea due to dumping of undigested starch products into the colon (Williams et al., 2015). Thus, dietary phenolic compounds as natural inhibitors, with only partial inhibition property, could be applied to inhibit starch digestive enzymes, while largely or completely slowly digesting starch, for controlling the postprandial blood glucose level without the side effects (Forester, Gu, & Lambert, 2012). Flavonoids as natural inhibitors have different inhibition property toward starch digestive enzymes based on their chemical backbone ring structure. The double bond between C2 and C3 on the C-ring of flavonoids plays an important role to inhibit α-amylases, causing π-π interaction. Meanwhile, the hydroxyl group at C3 is related to inhibition of α-glucosidases.

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Table 2-1. Classification of starch digestive enzymes in CAZy sustem

	Glycoside Hydrolase (GH)	
	α-Amylase	α-Glucosidase
Clan	H	D
Family	13	31
Subfamily	24	1
Catalytic mechanism	retaining	retaining
Catalytic domain fold	$(\beta/\alpha)_8$-barrel	$(\beta/\alpha)_8$-barrel

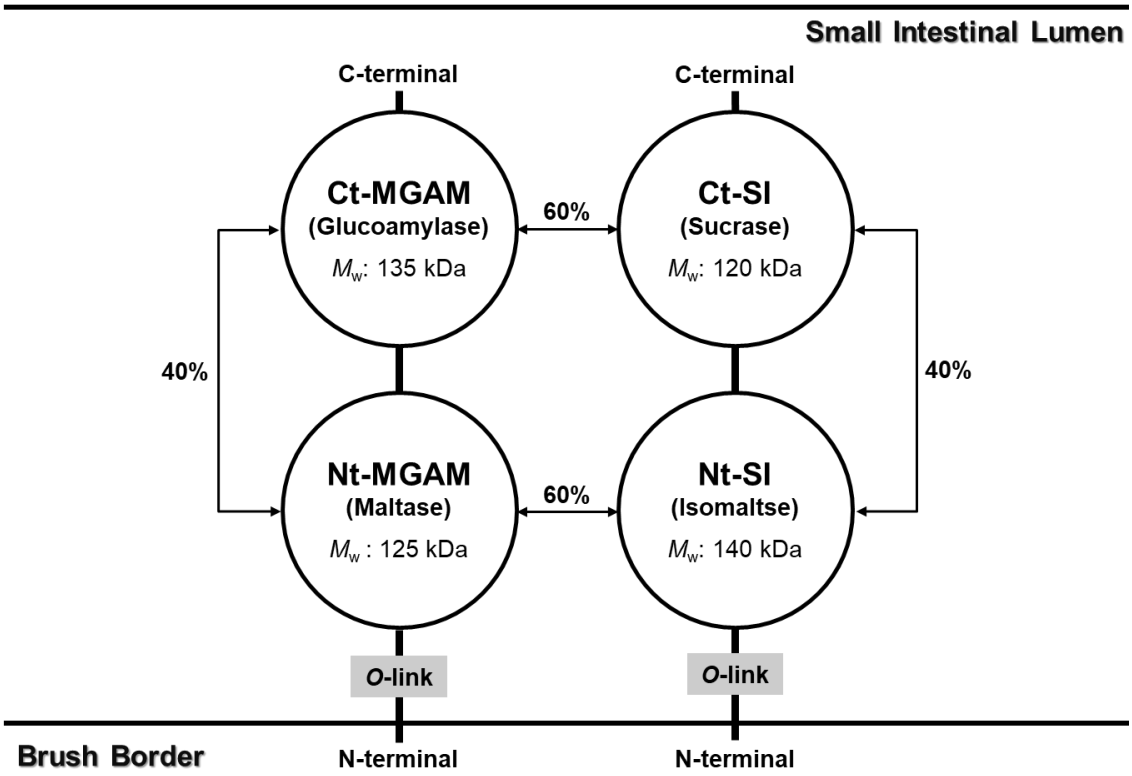

Figure 2-1. Glucosidases protein complex

CHAPTER 3. STARCH DIGESTED PRODUCT ANALYSIS BY HPAEC REVEALS STRUCTURAL SPECIFICITY OF FLAVONOIDS TO INHIBIT MAMMALIAN ALPHA-AMYLASE AND ALPHA-GLUCOSIDASES

3.1 Abstract

An accurate high-performance anion-exchange chromatography (HPAEC) method is presented to measure the inhibition property of flavonoids against mammalian starch digestive enzymes, because flavonoids interfere with commonly used 3,5-dinitrosalicylic acid (DNS) and glucose oxidase/peroxidase (GOPOD) methods. Eriodictyol, luteolin, and quercetin increased absorbance values (without substrate) in the DNS assay and, with substrate, either overestimated or underestimated values in the DNS and GOPOD assays. Using a direct HPAEC measurement method, flavonoids showed different inhibition properties against α-amylase and α-glucosidases, showing different inhibition constants (K_i) and mechanisms. The double bond between C2 and C3 on the C-ring of flavonoids appeared particularly important to inhibit α-amylase, while the hydroxyl group (OH) at C3 of the C-ring was related to inhibition of α-glucosidases. This study shows that direct measurement of starch digestion products by HPAEC should be used in inhibition studies, and provides insights into structure-function aspects of polyphenols in controlling starch digestion rate.

Keywords: Starch digestive enzymes, Inhibition, Flavonoids, HPAEC, Ring structure

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3.2 Introduction

Diabetes has become one of the principal diet-related metabolic diseases in the world (Mathers & Loncar, 2006) with an incidence of over 400 million people (WHO, 2016). The number of individuals with type 2 diabetes is estimated to increase to 642 million adults by 2040 (Ogurtsova, da Rocha Fernandes, Huang, Linnenkamp, Guariguata, Cho, et al., 2017). Control of postprandial glucose excursions is critical for diabetic patients.

Antidiabetic drugs, such as acarbose, miglitol, and voglibose, have been used to slow the rate of starch digestion in the gastrointestinal tract by inhibiting starch digestive enzyme activities for preventing hyperglycemia (Chiasson, Josse, Gomis, Hanefeld, Karasik, Laakso, et al., 2003). Because these drugs substantially inhibit α-amylase and α-glucosidases, they often result in side effects, such as diarrhea and discomfort, that result from the dumping of the undigested starch fraction into the colon (Chiasson, Josse, Gomis, Hanefeld, Karasik, & Laakso, 2002). An alternative strategy is to use less strong inhibitors of the starch-degrading enzymes to modulate glucose entry into the body, but with more complete digestion of starch (Simsek, Quezada-Calvillo, Ferruzzi, Nichols, & Hamaker, 2015). Considerable attention in this area has been directed toward use of food-based polyphenols (e.g. epigallocatechin gallate, myricetin, and quercetin) as natural starch digestive enzyme inhibitors to reduce postprandial glycemic response (Forester, Gu, & Lambert, 2012; Tarling, Woods, Zhang, Brastianos, Brayer, Andersen, et al., 2008; Zhang, Dong, Guangyong, Yuan, Tang, & Wang, 2018).

In vitro methods that have been used to determine the inhibition properties of dietary polyphenols on α-amylase and the α-glucosidases are based on colorimetric measurement of the released product amounts from substrates during starch digestive enzyme reactions by dinitrosalicylate (DNS) and glucose oxidase/peroxidase (GOPOD) assays (Miller, 1959; Trinder, 1969). The DNS assay is the most commonly used method for quantifying the reducing sugars digested from substrate by α-amylase activity (Karim, Holmes, & Orfila, 2017; Shobana, Sreerama, & Malleshi, 2009; Tan, Chang, & Zhang, 2017). The free carbonyl group (C=O) on the reducing sugar participates in the reduction of 3,5-dinitrosalicylic acid in an alkaline solution to produce the orange-red-colored 3-amino-5-nitrosalicylic acid. In the GOPOD assay, glucose as the digested product of α-glucosidase action is measured using GOPOD reagent. The hydrogen peroxide (H_2O_2) produced via the glucose oxidation by glucose oxidase reacts with *p*-hydroxybenzoic acid and 4-

aminoantipyrine by peroxidase to generate a quinoneimine dye that is pink. Dietary polyphenols are well known as antioxidants and free radical scavengers, and have been shown to interfere in the redox reaction of the DNS and GOPOD colorimetric methods (Nyambe-Silavwe, Villa-Rodriguez, Ifie, Holmes, Aydin, Jensen, et al., 2015; Ruch, Cheng, & Klaunig, 1989). Additionally, the DNS assay is influenced by the molecular size of oligosaccharides, resulting in overestimated reducing power (Shao & Lin, 2018). Thus, the presence of dietary polyphenols in the DNS and GOPOD assay systems is likely a confounding factor in evaluating their inhibition properties on starch digestive enzyme activities.

Flavonoids, a major polyphenol group with reported starch digestive enzyme inhibition properties, are present in wide variety of dietary sources, such as fruits, vegetables, and cereals (Williamson, 2013). Flavonoids consist of two benzene rings linked by a heterocyclic six-membered pyrone ring, and are classified as flavanones, flavanols, flavones, flavonols, and isoflavones based on variations in the C-ring (Van Acker, Van Den Berg, Tromp, Griffioen, Van Bennekom, Van Der Vijgh, et al., 1996). Chemical structure of flavonoids is related to their biological activity (Williamson & Clifford, 2010), and the relationship between flavonoid structure and starch enzyme inhibition has received significant attention for modulating starch digestion rate. Structural effects of the C-ring toward α-amylase and the α-glucosidases inhibition have been previously investigated with varied results. For instance, Tadera et al. (2006) noted that the hydroxylation at R3 position of the flavonoid structures (Supplementary Fig. S1) act as a negative factor in the inhibition of α-amylase, whereas the hydroxyl group at C3 on the C-ring enhances the inhibition property of flavonoids for α-glucosidases. However, it has been shown that an additional hydroxyl group at C3 on the C-ring of flavonoids boosts the inhibition property for both starch enzyme activities (Wang, Du, & Song, 2010). In order to systematically study the polyphenol structure and inhibition function relationship, it is critical to have a reliable and accurate enzyme assay that is free from interference of target polyphenols of interests.

Therefore, in the present study we showed problems using selected flavonoids with the DNS and GOPOD assays and compared those results with direct measurement of starch digestion products by high-performance anion-exchange chromatography (HPAEC). Using flavonoids, chosen based on the degree of hydroxylation and planarity of the C-ring (eriodictyol, luteolin, and quercetin; Supplementary Fig. S1), structural specificities were found for α-amylase and the α-glucosidases inhibition that were not shown with the colorimetric methods. A new analytical

technique is proposed using HPAEC that may provide better insights into structural aspects of flavonoids for the inhibition of α-amylase and the α-glucosidases, and how to apply dietary phenolic compounds for controlling glycemic response.

3.3 Materials and methods

3.3.1 Classification in Carbohydrate-Active enZymes system (CAZy)

Starch digestive enzymes, α-amylase and α-glucosidase, are all members of glycosyl hydrolase (GH) in Carbohydrate-Active enZymes system (CAZy) (Nucleic acids research 2009), Maltodextrin (DE 10) and waxy corn starch (Ingredion, Westchester, IL, USA), and glucose, maltose, maltotriose, isomaltose, sucrose (purity 99%, Sigma-Aldrich, St. Louis, MO, USA), maltotetraose (purity > 90%, Megazyme, IL, USA), and maltopentaose (purity > 90%, Carbosynth, San Diego, CA, USA) were utilized as substrates and standard sugars. Porcine pancreatic α-amylase (Type VI-B, Molecular mass: 51-54 kDa) and rat intestinal acetone powder (Sigma-Aldrich, St. Louis, MO, USA) were used to prepare enzyme solutions for this study. Eriodictyol, luteolin (purity 95-98%, Sigma-Aldrich, St. Louis, MO, USA), and quercetin (purity 98%, Tocris Bioscience, Minneapolis, MN, USA) were used for enzyme inhibition assay.

3.3.2 Absorbance value of substrate with flavonoids

Eriodictyol, luteolin, and quercetin were applied to evaluate the confounding effects of flavonoids on DNS and GOPOD assays. Maltose and glucose solutions with a range of concentrations from 0.1 to 1.0 mg/mL in sodium phosphate buffer (100 mM, pH 6.9) were first mixed with 750 μM/mL dietary flavonoids in dimethyl sulfoxide (DMSO) due to their low solubility in the buffer, and then incubated in a thermomixer (Eppendorf, Hauppauge, NY, USA) at 37 ^{o}C and 800 rpm for 15 min. The mixtures of substrates with flavonoids and flavonoids alone were reacted with DNS and GOPOD reagents, respectively, and absorbances were read on a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The mixed glucose solutions with flavonoids were further diluted 100 times with water, and then 50 μL, passed through a 0.45 nm nylon syringe filter, were injected into a HPAEC (Dionex, Sunnyvale, CA, USA) equipped with a CarboPac PA-1 and an electrochemical detector to measure the glucose concentration. A solution

containing 200 mM sodium hydroxide was used as the mobile phase and flow rate was set at 1 mL/min. All treatments were performed in triplicate.

3.3.3 Substrate selection for HPAEC assay

A 1.0% (w/v) solution of waxy corn starch, maltodextrin (DE 10), and maltopentaose for α-amylase and 2.5% (w/v) solution of maltose, isomaltose, and sucrose for α-glucosidases were used to investigate their hydrolysis properties against starch digestive enzymes for selecting optimal substrate for HPAEC assay. Porcine pancreatic α-amylase and rat intestinal acetone powder were mixed with sodium phosphate buffer (100 mM, pH 6.9) to make a 1.0% (w/v) solution of α-amylase and 10% (w/v) solution of α-glucosidases, and then placed at 4 °C for 30 min. The chilled solutions were centrifuged at 4 °C and 8,000 rpm for 15 min, and the supernatants were collected for use as enzyme solutions. The different substrates were reacted with the enzyme solutions using the thermomixer at 37 °C and 800 rpm for 60 min. The sample tubes were placed in boiling water to terminate the enzyme reactions and the resulting products, digested from different substrates by enzyme activities, were measured by the DNS and GOPOD methods (DNS & GOPOD). Furthermore, Michaelis-Menten kinetic parameters of the starch digestive enzymes with different substrates were obtained (Eisenthal, Danson, & Hough, 2007) using SigmaPlot 12 (Systat Software, San Jose, CA, USA).

3.3.4 Inhibition property of flavonoids determined by colorimetric and HPAEC methods

Eriodictyol, luteolin, and quercetin were used to evaluate their inhibition properties against α-amylase and α-glucosidases measured by color-development reagents and HPAEC. Flavonoid solutions were prepared in DMSO in different concentrations from 31.25 to 750 μM/mL, which is within a range of physiological relevance (Williamson, 2013). The flavonoid solutions were pre-warmed with substrates, maltopentaose (1.0%, w/v) for α-amylase and maltose (2.5%, w/v) for α-glucosidases, in the thermomixer at 37 °C and 800 rpm for 15 min. The solutions were reacted with enzyme solutions, α-amylase (1.0%, w/v) and α-glucosidases (10%, w/v), at 37 °C and 800 rpm for 15 min, and then placed in boiling water to stop enzyme activities. The digested products were determined using DNS and GOPOD methods. The inhibition property of flavonoids was calculated as a percentage of the control as follows:

$$\text{Inhibition (\%)} = \frac{\text{Control}_{(\text{Abs})} - (\text{Sample}_{(\text{Abs})} - \text{Flavonoid}_{(\text{Abs})})}{\text{Control}_{(\text{Abs})}} \times 100$$

Additionally, the test solutions were diluted with water up to 500 times and filtered through a 0.45 nm nylon syringe filter prior to injection into the HPAEC to measure the digested product amounts. Two mobile phases, 120 mM sodium acetate in 200 mM sodium hydroxide and 200 mM sodium hydroxide, were used for profiling the released products from maltopentaose and maltose, respectively, using a 1 mL/min flow rate. The inhibition rate of flavonoids was calculated using the following equation:

$$\text{Inhibition (\%)} = \frac{\text{Control}_{(\text{Molecular mass})} - \text{Sample}_{(\text{Molecular mass})}}{\text{Control}_{(\text{Molecular mass})}} \times 100$$

3.3.5 Inhibition constant and mechanism of flavonoids against starch digestive enzymes

Solutions of eriodictyol, luteolin, and quercetin at different concentrations from 31.25 to 750 µM/mL were used to determine the inhibition constant (K_i) and mechanism of flavonoids toward starch digestive enzymes. Flavonoid solutions were mixed with substrates, maltopentaose from 12.1 to 30.2 mM for α-amylase and maltose from 35.1 to 280.5 mM for α-glucosidases, and then warmed and stirred in the thermomixer at 37 °C and 800 rpm for 15 min. The pre-warmed solutions were further reacted with α-amylase (1.0%, w/v) and α-glucosidases (10%, w/v) at 37 °C and 800 rpm for 15 min. Samples were then processed as described above (Sec. 2.4) for HPAEC analysis. Lineweaver-Burk plots were constructed for complexes of enzymes and flavonoids (Lineweaver & Burk, 1934) using the SigmaPlot 12.

3.3.6 Statistical analysis

All values were expressed as the mean ± standard deviation (SD) from $n = 3$ measurements. Differences across treatments were analyzed by one-way ANOVA using the post-hoc Tukey test using SAS v.9.4 software (SAS Institute, Cary, NC, USA) to form statistical groupings ($\alpha = 0.05$).

3.4 Results and discussions

3.4.1 Change in absorbance value of substrate with flavonoids

As shown in Figs. 1A and C, the presence of flavonoids in DNS and GOPOD mixtures led to changes in absorbances over a range of substrate concentrations, resulting in a rise in values for DNS and a drop in GOPOD assays ($\alpha = 0.05$). In comparison to the colorimetric methods, HPAEC results were not affected by added flavonoids at different substrate concentrations (Fig. 1E).

The addition of flavonoids to the DNS reagent mixture alone resulted in a marked increase in absorbance values with increasing concentration up to 750 µM/mL (Fig. 1B), which indicated a certain contribution of hydroxyl groups on phenolic rings with respect to the redox reaction via the DNS assay. This follows the work by Nyambe-Silavwe et al. (2015), who showed the number of reducing hydroxyl groups on flavonoids interferes with DNS reagent by participating in the chemical reaction to produce 3-amino-5-nitrosalicylic acid from the reduction of 3,5-dinitrosalicylic acid.

Conversely for the GOPOD assay, the inclusion of flavonoids in glucose solutions resulted in a decrease in absorbance values in the GOPOD assay, compared to glucose alone (Fig. 1C). A similar observation of decrease absorbance due to interference of flavonoids in GOPOD solutions was noted by Xu et al. (2012) and Pyner et al. (2017). Considering that the tested flavonoids had negligible interference with the GOPOD reagent (Fig. 1D), this indicates a direct interaction in the GOPOD assay either through intervention of flavonoids as an oxygen acceptor or scavenger in the redox reaction, or inhibition property against glucose oxidase and peroxidase to form the pink-colored quinoneimine dye.

Compared to the colorimetric methods, the HPAEC method showed no interference when flavonoids were added over a range of glucose concentrations (Fig. 1E), or when tested alone (Fig. 1F). Based on the observations described above, the HPAEC chromatographic method appears to be a more accurate way to determine substrate concentration and avoid flavonoid interferences in the DNS and GOPOD assays.

3.4.2 Choice of optional substrate for the HPAEC assay

The susceptibility of substrate to α-amylase digestion is intimately related to the degree of polymerization or chain length of the α-glucan, because α-amylase prefers a binding mode in which all five binding subsites are filled with substrate (Brayer, Sidhu, Maurus, Rydberg, Braun, Wang, et al., 2000; Ramasubbu, Paloth, Luo, Brayer, & Levine, 1996). Robyt & French (1963) noted that the products formed by the initial α-amylase action hinder the latter formation of the enzyme-substrate complex by blocking the binding sites. Additionally, the branched products degraded from amylopectin have insufficient glucose units between the branch points to produce the enzyme-substrate complex.

Considering the five substrate binding subsites of α-amylase and the potential interference of activity by the degraded compounds themselves, maltopentaose with five linear glucose units was found to be a better substrate than maltodextrin or starch as it had higher substrate specificity (K_{cat}/K_m = 1.31) and hydrolysis rate (Table 1 and Supplementary Fig. S2A). Thus, maltopentaose was applied as the substrate to the HPAEC method to investigate the inhibition property of flavonoids against α-amylase. Furthermore, the extract from rat intestinal powder showed higher ability to digest linear α-1,4 maltose with K_{cat}/K_m of 2.71, compared to sucrose and isomaltose (K_{cat}/K_m of 0.03 and 0.02, respectively; Table 1 and Supplementary Fig. S2B). In this study, maltose with higher hydrolysis rate was used as the substrate to evaluate the inhibition property of flavonoids toward mucosal α-glucosidase activity by the HPAEC method.

3.4.3 Substrate degradation by starch digestive enzymes with flavonoids

As shown in Figs. 2A and B, the products degraded from maltopentaose and maltose by starch digestive enzymes with flavonoids are profiled using the HPAEC system. Pancreatic α-amylase hydrolyzed maltopentaose into smaller units from glucose to maltotetraose with the highest released amounts of maltose, followed by maltotriose, glucose, and maltotetraose (Fig. 2A). This was expected, as mammalian α-amylase has its cleavage site between the second and third subsites of the five substrate binding sites, thus hydrolyzing maltopentaose mainly to maltose and maltotriose. Glucose as a minor product is generated by a slower secondary hydrolysis of the digested products by the initial α-amylase action (Robyt & French, 1970; Seigner, Prodanov, & Marchismouren, 1987; Lin, Ao, Quezada-Calvillo, Nichols, Lin, & Hamaker, 2014). The addition

of flavonoids exhibited reduced peak area of the products hydrolyzed by α-amylase (Fig. 2A), compared to the control without flavonoids. Luteolin and quercetin showed greater decrease in peak areas than eriodictyol. It was previously shown that flavonoids bind in the active site of α-amylase, and that differences in structures affect binding affinities and inhibition properties (Xiao, Ni, Kai, & Chen, 2013). Piparo et al. (2008) reported that quercetagetin, a flavonol with an additional hydroxyl group at R6 on the A-ring of quercetin (Supplementary Fig. S1), lies parallel on the substrate binding subsites of α-amylase, and then forms π-stacking interaction between the B-ring of quercetagetin and α-amylase catalytic residue Tyr^{59} (tyrosine). Additionally, hydroxyl groups at R3' and R4' on the B-ring of flavonoids bind with Asp^{197} (aspartic acid) and Glu^{233} (glutamic acid) at the third substrate binding site, subsite-1 (Williams, Zhang, Caner, Tysoe, Nguyen, Wicki, et al., 2015). It means that flavonoids block the main catalytic site of α-amylase by bonding to the amino acid residues, resulting in the inhibition of α-amylase activity. The flavonoids used in this study with the double bond in the C-ring of luteolin and quercetin showed higher α-amylase inhibition property that was apparently targeted to the same subsites, as maltose and maltotriose products were reduced while glucose and maltotetraose remained similar to the control.

The three flavonoids also exhibited a lower peak of the glucose released from maltose by α-glucosidases than the control in the absence of flavonoids. The quercetin treatment had a lower glucose peak, compared to eriodictyol and luteolin which showed the similar reduced peaks. This is likely the result of different inhibition activities for the α-glucosidases due to the presence or absence of the hydroxyl group at C3 on the C-ring of flavonoids. Similar observations of the hydroxylation at C3 positively influencing inhibition property of kaempferol on rat intestinal α-glucosidases were noted by Tadera et al. (2006) and Wang et al. (2010). An explanation for the reduced peaks of the products released from maltopentaose and maltose, is that the carbon-carbon double bond between C2 and C3 on flavonoid's ring structure enhances the inhibition property for α-amylase, while the hydroxyl group at R3 position on the C-ring affects α-glucosidase inhibition. Not only was the HPAEC method an accurate and better way to estimate the inhibition property of flavonoids with specific substrates by directly measuring the digested products, it provides a more mechanistic view of how polyphenols inhibit enzymes.

3.4.4 Comparison of inhibition properties using colorimetric and HPAEC methods

Differences in the evaluation of inhibition properties between the colorimetric and HPAEC assay systems are shown in Table 2 and Supplementary Fig. S3. The type of inhibition via the colorimetric methods differed from the HPAEC method, with lower values in DNS and higher values in GOPOD.

In the DNS assay, the three flavonoids exhibited weak inhibition, under 14%, against α-amylase, and significant differences across the three flavonoids were not observed at $\alpha = 0.05$. For the HPAEC method, luteolin and quercetin inhibited around 72.5% of α-amylase activity, whereas eriodictyol displayed the lowest 40.4% inhibition. Differences in inhibition among the three flavonoids formed two different statistical groups at $\alpha = 0.05$ levels. Underestimation of degree of inhibition of flavonoids in the DNS assay system is likely the result of overproduced 3-amino-5-nitrosalicylic acid, as the indicator for the released reducing sugars, due to participation of flavonoids in the reduction of 3,5-dinitrosalicylic acid (Nyambe-Silavwe, Villa-Rodriguez, Ifie, Holmes, Aydin, Jensen, et al., 2015).

The flavonoids in the GOPOD method showed higher degree of inhibition against α-glucosidases than the HPAEC system, with quercetin having the highest inhibition (73.3%) followed by eriodictyol (35.5%) and luteolin (26.0%). Statistical analysis sorted the flavonoids into three different groups ($\alpha = 0.05$). For the HPAEC method, quercetin had the highest inhibition of 50.4% against the α-glucosidases, with eriodictyol and luteolin displaying lower and similar inhibitions of 24.2 and 24.8%, giving two different statistical groups at $\alpha = 0.05$ levels. It seems likely that the overestimation of flavonoid inhibition via the GOPOD assay is related to the interaction between flavonoids and H_2O_2, the substrate of peroxidase, to produce the pink-colored quinoneimine dye (Trinder, 1969).

Based on these observations, it is noteworthy that, by HPAEC analysis, the flavonoids used in this study showed different inhibition against the two starch degrading enzymes. This may be due to the different protein structures of α-amylase and α-glucosidases, belonging to the glycosyl hydrolase families 13 and 31 (GH13 and GH31) (Williams, Zhang, Caner, Tysoe, Nguyen, Wicki, et al., 2015). Clearly, direct measurement of the products degraded from substrates by starch digestive enzymes using HPAEC is a more accurate way to investigate the inhibition property of

flavonoids and would allow for more accurate determination of subtle-structure function relationships.

3.4.5 Interference of flavonoids on colorimetric methods

As described above (Sec. 3.1), flavonoids act as antioxidant or oxygen acceptor in the redox reactions via the colorimetric methods, causing change in absorbance values. In the DNS method, the free carbonyl group (C=O) at C1 of the reducing sugar is oxidized in the reduction of 3,5-dinitrosalicylic acid to produce the orange-red-colored 3-amino-5-nitrosalicylic acid, as the indicator for the reducing sugar. Coincidentally, flavonoids introduced in the DNS reagent mixture also participate in the reduction of 3,5-dinitrosalicylic acid, and then produce more orange-red-colored 3-amino-5-nitrosalicylic acid (Fig. 3A), resulting in higher absorbance values than the actual amount of reducing sugars (Fig. 1A). In addition, the number of hydroxyl groups on flavonoids is related with the increase in absorbances in the DNS assay system (Nyambe-Silavwe, Villa-Rodriguez, Ifie, Holmes, Aydin, Jensen, et al., 2015).

In the GOPOD method, the glucose degraded from substrate by α-glucosidases is oxidized to gluconic acid by glucose oxidase, producing hydrogen peroxide (H_2O_2), and then the produced H_2O_2 is further used by peroxidase to convert 4-aminoantipyrine and *p*-hydroxybenzoic acid into the pink-colored quinoneimine. Due to the antioxidant capacity or inhibition property of flavonoids, flavonoids become a confounding factor in the formation of quinoneimine, the color forming compound of the assay (Fig. 3B). Flavonoids are able to scavenge the H_2O_2 generated by glucose oxidase action, decreasing the potential for 4-aminoantipyrine to be oxidized to generate quinoneimine. Ruch et al. (1989) previously reported that the presence of catechins from green tea in H_2O_2 solution reduces H_2O_2 concentration due to their antioxidant activity toward H_2O_2, as well as reduce the superoxide radical ($O_2^{\cdot-}$). Additionally, flavonoids may compete against *p*-hydroxybenzoic acid for reacting with H_2O_2 by peroxidase, acting as an alternative oxygen acceptor (Trinder, 1969). Furthermore, Xu et al. (2012) and Wong & Huang (2014) proposed that the enzyme activities of glucose oxidase or peroxidase can be inhibited by flavonoids, causing interference in the glucose measurement.

3.4.6 Selective inhibition property of flavonoids against starch digestive enzymes

As shown in Figure 4, the flavonoids used in this study selectively inhibited the starch digestive enzymes, resulting in a different inhibition constant (K_i) and mechanism of flavonoids against α-amylase and α-glucosidases.

Both of luteolin and quercetin had lower inhibition constants (K_i of 0.22 and 0.18 mM, respectively) for α-amylase, compared to eriodictyol (K_i = 5.09 mM). Furthermore, luteolin and quercetin showed a competitive inhibition mechanism against α-amylase, whereas eridodictyol inhibited α-amylase in noncompetitive behavior. Considering the differences in the ring structures of flavonoids (Supplementary Fig. S1), the double bond between C2 and C3 on the C-ring of luteolin and quercetin is implicated in the inhibition of α-amylase, allowing easy access of the flavonoids for bonding to the catalytic residue Tyr^{59} (tyrosine) on substrate third binding subsite to form the π-π interaction between the ligand and protein (Williams, Zhang, Caner, Tysoe, Nguyen, Wicki, et al., 2015). Additionally, the competitive inhibition mechanism of luteolin and quercetin for α-amylase indicates that the two flavonoids occupy substrate binding subsites by binding with Asp^{197} (aspartic acid) and Glu^{233} (glutamic acid) at the catalytic center subsite (Piparo, Scheib, Frei, Williamson, Grigorov, & Chou, 2008), resulting in the observed reduction in maltose and maltotriose products and enzyme inhibition (Fig. 2A).

Quercetin showed a lower inhibition constant (K_i) of 0.11 mM for the α-glucosidases, behaving in competitive manner to inhibit α-glucosidases, whereas eriodictyol and luteolin had K_i of 2.59 and 2.69 mM, respectively, with a noncompetitive inhibition mechanism. Compared to α-amylase inhibition, it seems likely that the hydroxyl group at C3 on the C-ring of flavonoids is more important than the double bond between C2 and C3 of the C-ring for inhibiting the α-glucosidases. It stands to reason that the selective and different inhibition property of flavonoids against the two starch digestive enzymes is due to their different protein structures, as α-amylase and α-glucosidases belong to the GH13 and GH31, respectively.

3.5 Conclusions

The conventional way to measure the inhibition property of flavonoids against starch digestive enzymes is to use colorimetric methods, such as DNS and GOPOD assays, by quantifying the amounts of degraded products. However, this analytical approach is not valid,

because flavonoids become a confounding factor in the redox reactions of these colorimetric methods due to their ability to act as oxygen acceptors, which causes change in absorbance values. Additionally, flavonoids may inhibit glucose oxidase or peroxidase in the GOPOD assay, as well as the deep color of some flavonoids, such as gossypetin or morin, can interfere in the colorimetric methods. Here, we showed that direct measurement of the digested products using HPAEC is an accurate and precise way to investigate the inhibition property of flavonoids.

Using the HPAEC method, structural specificity of flavonoids to inhibit α-amylase and α-glucosidases was revealed that was not shown in the colorimetric methods. Eriodictyol, luteolin, and quercetin were chosen based on structures that differ only in the C-ring. The double bond between C2 and C3 on the C-ring of flavonoids was particularly important for inhibition of α-amylase, which leads to a π-π interaction between flavonoids and α-amylase, whereas addition of a hydroxyl group at C3 of the C-ring was related to the inhibition of the α-glucosidases. These structural specificities of flavonoids for α-amylase and α-glucosidases are likely the result of different protein structures of the two starch digestive enzymes, as α-amylase and α-glucosidases belong to different families, GH13 and GH31. The finding of structural specificity of flavonoids to mammalian starch digestive enzymes implies that starch digestion rate could be selectively inhibited by certain compounds with higher level of specificity toward α-amylase or α-glucosidases. Flavonoids, with mild inhibitory properties compared to the strong drug-type inhibitors (e.g. acarbose) and that are specific to different starch degradation enzymes, could produce slow starch digestion profiles and result in fewer undesirable side effects.

3.6 References

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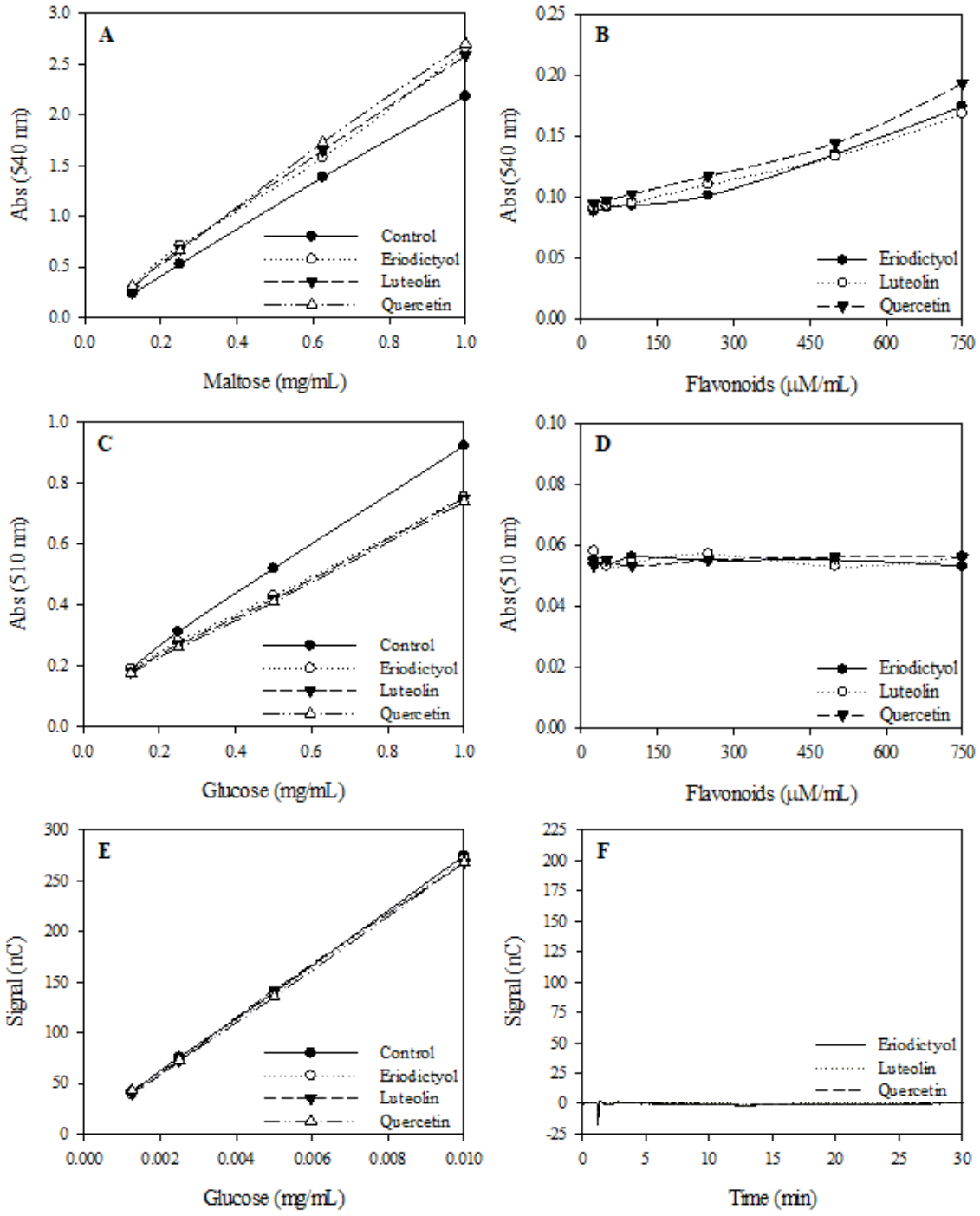

Figure 3-1. Effects of flavonoids on colorimetric methods. Absorbances of mixtures of maltose and flavonoids (A) and flavonoids alone (B) measured by the DNS method; absorbances of mixtures of glucose and flavonoids (C) and flavonoids alone (D) measured by the GOPOD method; peak signals of mixtures of glucose and flavonoids (E) and flavonoids alone (F) measured by the HPAEC method

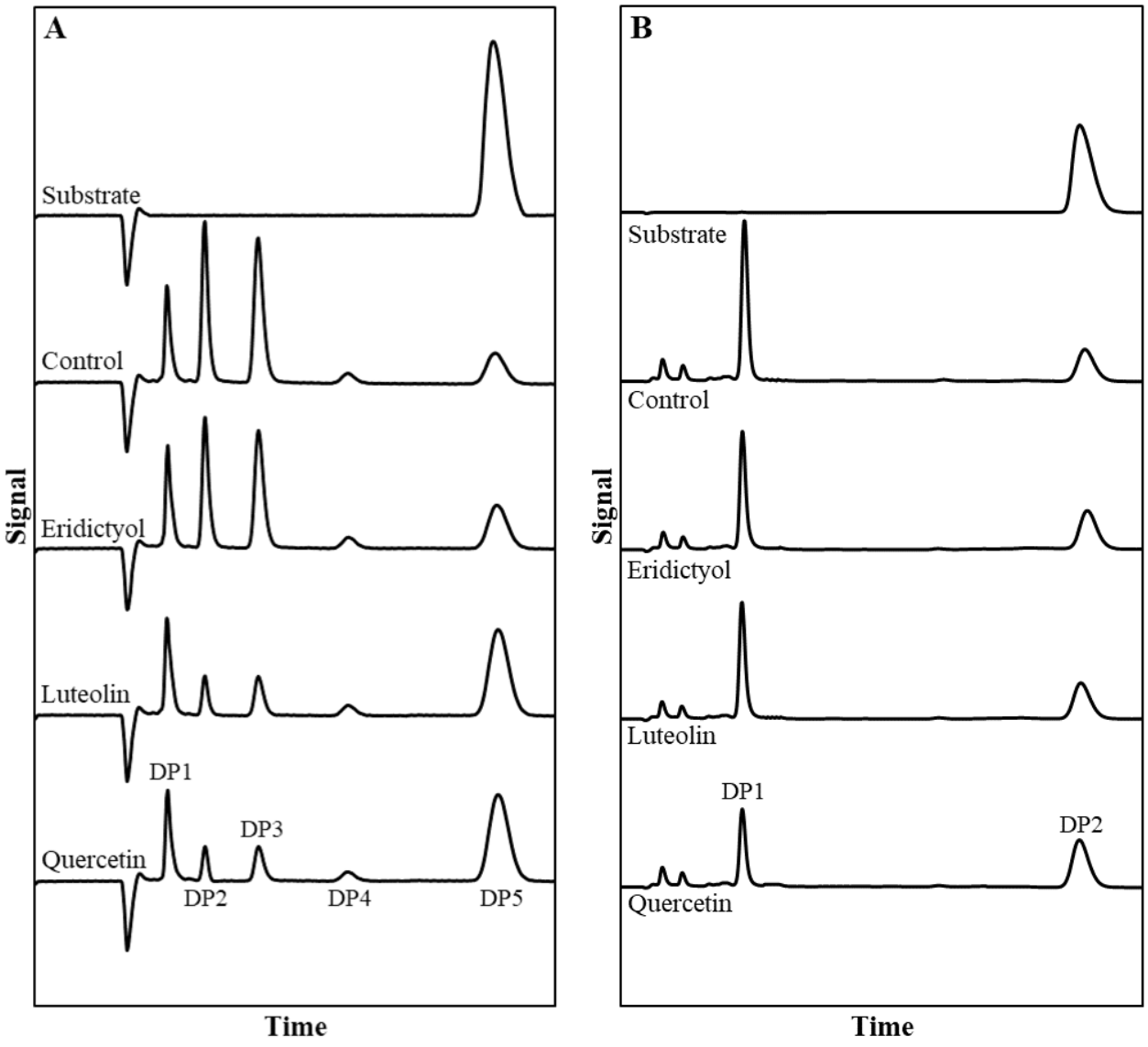

Figure 3-2. Profile of substrate (maltopentaose, maltose) degradation by starch digestive enzymes (α-amylase, α-glucosidases) in the presence of flavonoids using HPAEC. Degradation of substrates by α-amylase (A) and α-glucosidases (B) with flavonoids. DP1: glucose; DP2: maltose; DP3: maltotriose; DP4: maltotetraose; DP5: maltopentaose

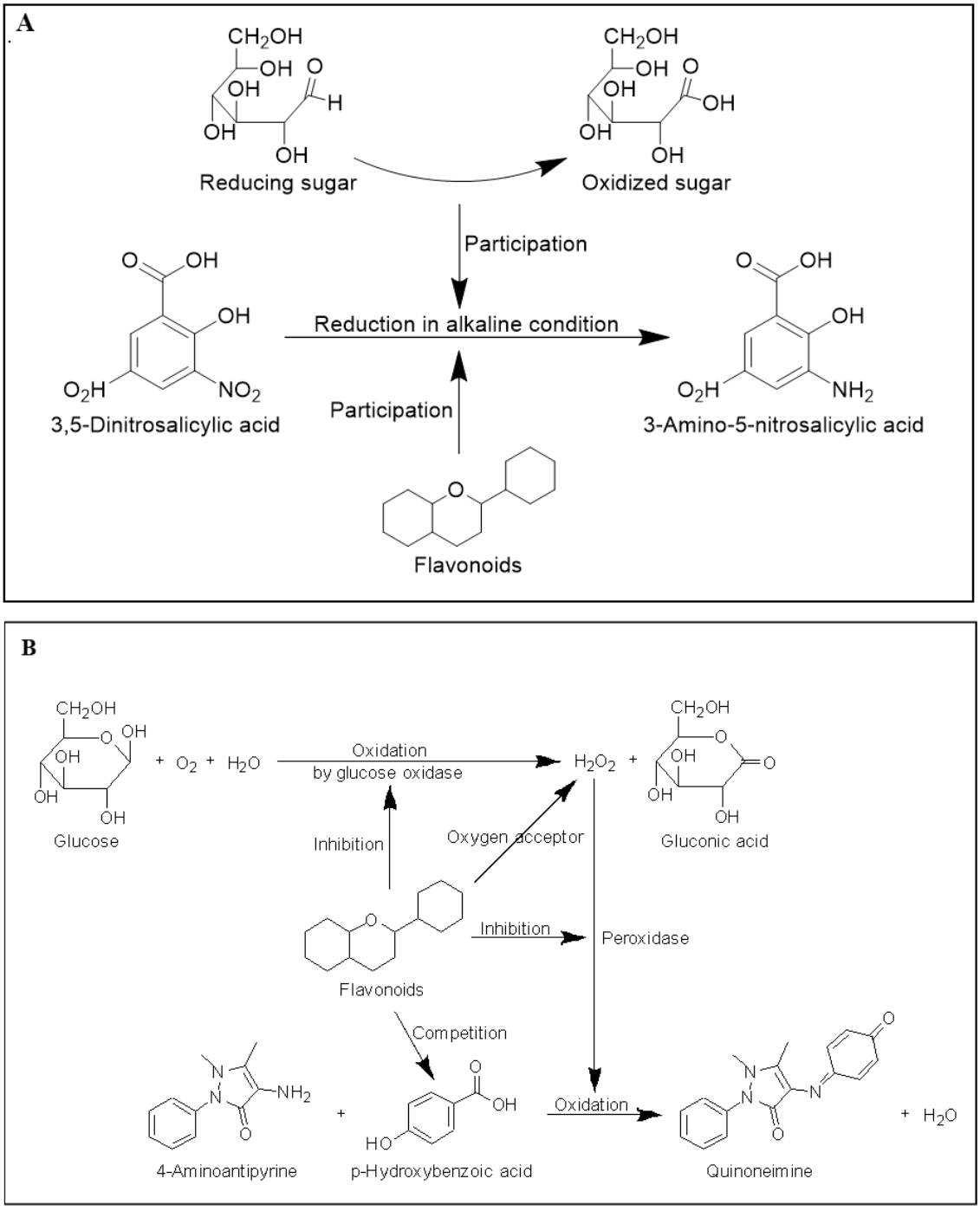

Figure 3-3. Schematic diagram for potential interruptions of flavonoids on colorimetric methods. Interferences of flavonoids in DNS (A) and GOPOD (B) assays

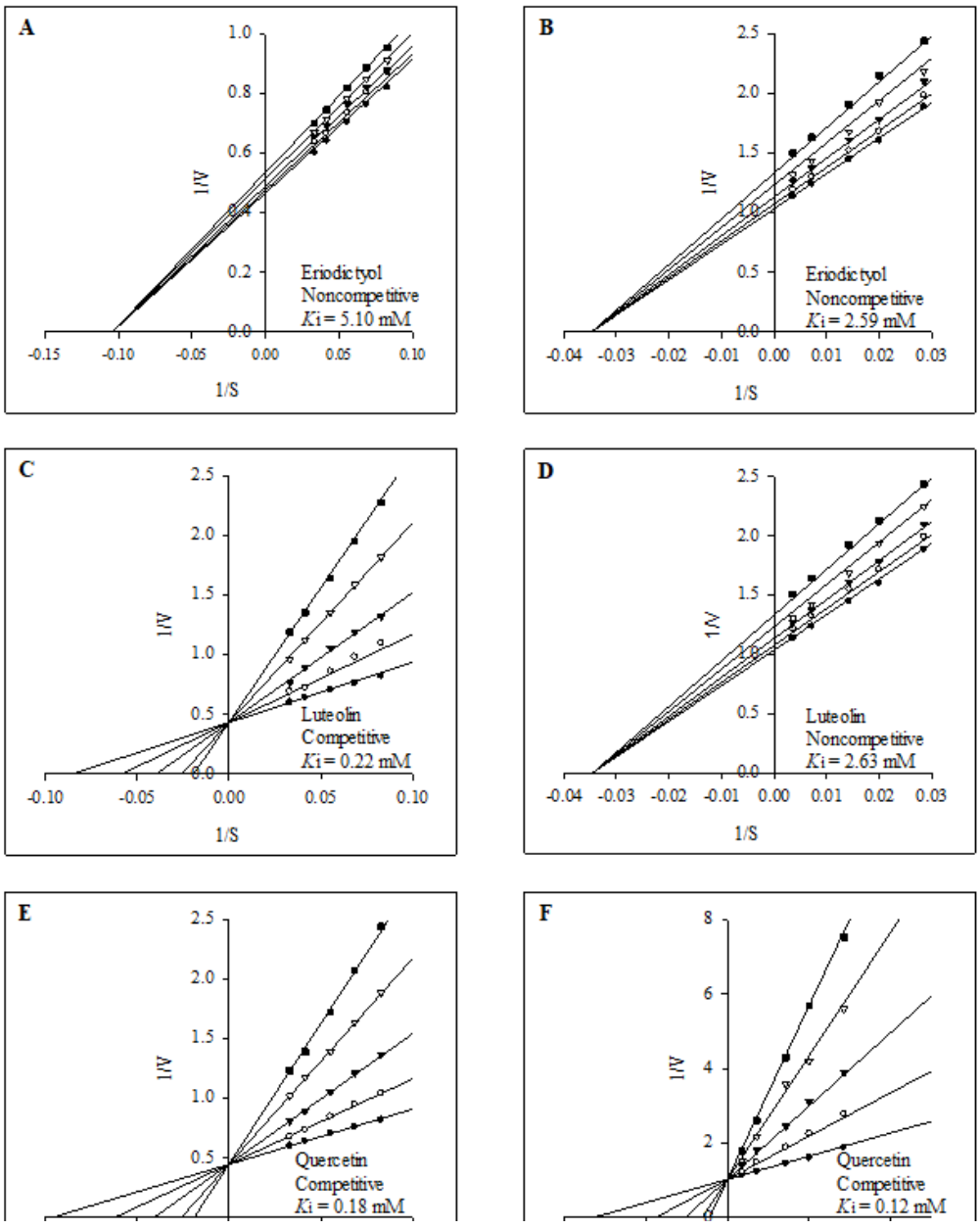

Figure 3-4. Inhibition mechanism and constant (K_i) of flavonoids for starch digestive enzymes. Inhibition properties of eriodictyol for α-amylase (A) and α-glucosidases (B); luteolin for α-amylase (C) and α-glucosidases (D); quercetin for α-amylase (E) and α-glucosidases (F).

Table 3-1. Kinetic parameters of different substrates toward starch digestive enzymes. *WCS: Waxy Corn Starch; MD: Maltodextrin; DP5: Maltopentaose, ** K_m: Substrate concentration at which the reaction velocity is half-maximal; K_{cat}: Catalytic constant for the conversion of substrate to product; K_{cat}/K_m: Catalytic efficiency

	α-Amylase			α-Glucosidases		
	WCS*	MD*	DP5*	Maltose	Sucrose	Isomaltose
K_m (mM)**	6.60	5.50	4.50	3.46	15.61	19.11
K_{cat} (s^{-1})**	1.77	3.47	5.89	9.39	0.52	0.32
K_{cat}/K_m**	0.27	0.63	1.31	2.71	0.03	0.02

Table 3-2. Comparison of inhibition properties (%) of flavonoids determined by colorimetric and HPAEC methods. Different letters denote significant differences at $\alpha = 0.05$.

	α-Amylase		α-Glucosidases	
	DNS assay	HPAEC	GOPOD assay	HPAEC
Eriodictyol	12.4 ± 4.1^{A}	40.4 ± 0.9^{b}	35.5 ± 2.3^{B}	24.2 ± 0.8^{b}
Luteolin	11.9 ± 5.1^{A}	72.2 ± 0.8^{a}	26.0 ± 3.6^{C}	24.8 ± 0.4^{b}
Quercetin	13.2 ± 3.6^{A}	73.2 ± 0.7^{a}	73.3 ± 1.9^{A}	50.4 ± 1.2^{a}

CHAPTER 4. STRUCTURAL SPECIFICITYOF FLAVONOIDS TO INHIBIT STARCH DIGESTIVE ENZYMES

4.1 Abstract

Fourteen different flavonoids were tested to investigate the structural specificity of flavonoids to inhibit starch digestive enzymes, human pancreatic α-amylase and the N-terminal subunit of the α-glucosidase maltase-glucoamylase (MGAM), using an HPAEC system. Flavonoids showed different inhibition properties against α-amylase and α-glucosidases based on their chemical ring structures. The double bond between C2 and C3 on the flavonoid ring structure was found to play a critical role in inhibiting α-amylase activity, forming π-staking interaction between the flavonoid and α-amylase. Meanwhile, the hydroxyl group at C3 was intimately related to the inhibition of the α-glucosidases. The hydroxyl groups at B3 and A5 were also observed as key hydroxyl groups to inhibit starch digestive enzymes, leading to the binding of flavonoids in the vicinity of the catalytic active sites on the enzyme surfaces. These different inhibition properties of starch enzymes by flavonoids is likely the result of different protein structures of the two starch digestive enzymes, as α-amylase has the wide and shallow substrate binding site and the α-glucosidases contain narrow and deep pockets on the protein surface leading to the catalytic sites.

Keywords: Starch digestive enzymes, Inhibition, Flavonoid ring structure

4.2 Introduction

It is estimated that about 400 million people suffer from type 2 diabetes due to pancreatic β-cell dysfunction and insulin resistance with impaired glucose tolerance (Mathers & Loncar, 2006). The numbers are predicted to continue to rise. Antidiabetic drugs, acarbose, miglitol, and voglibose, are used in the treatment of type 2 diabetes (Chiasson, Josse, Gomis, Hanefeld, Karasik, & Laakso, 2002), which regulate high postprandial glucose spike in the blood by slowing starch digestion rate. Unfortunately, they often cause certain side effects such as diarrhea and discomfort, that result from dumping of the undigested starch fraction into the colon (Lee et al., 2012). The considerable restriction of starch digestion is from poorer specificity of the compounds for the enzymes coupled with strong inhibition, causing the substantial inhibition or even shutting down all enzyme activities. Hence, selective and partial inhibition of starch digestive enzymes with higher specificity could be used to improve postprandial blood glucose response by inhibiting enzymes without the adverse side effects (Lim, Zhang, Ferruzzi, & Hamaker, 2019; Williams et al., 2015). Phenolic compounds are present in dietary sources such as fruits, vegetables, and cereals, and have been shown to be inhibitory for starch digestion enzymes, and with selectivity towards the different enzymes. Tarling *et al*. (2008) extracted a compound from *Crocosmia crocosmiiflora*, which has a higher specific inhibition toward human pancreatic α-amylase with lower or even no inhibition of the α-glucosidases. Furthermore, four different dietary phenolic compounds (chlorogenic acid, EGCG, catechin, caffeic acid, and gallic acid) selectively inhibited the individual subunits of the α-glucosidases, MGAM and SI (Simsek, Quezada-Calvillo, Ferruzzi, Nichols, & Hamaker, 2015). In Chapter 3, it was noted that luteolin has a higher selectivity for porcine pancreatic α-amylase inhibition, while eriodictyol showed a selective inhibition of the α-glucosidases from rat intestinal powder, and these differences were based on their chemical ring structures (Lim, Kim, Shin, Hamaker, & Lee, 2019). Additionally, Kim et al. (2000) reported that the tested flavonoids, amentoflavone, genestin, hyperin, and inkgetin, showed selective and different inhibition properties against porcine pancreatic α-amylase and yeast α-glucosidases.

The double bond between C2 and C3 plays a key role to selectively inhibit α-amylase, leading to a π-π interaction between flavonoids and α-amylase due to a higher degree of planarity on flavonoid ring structure, while the hydroxyl group at C3 is intimately related to target the inhibition of α-glucosidases (Lim, Zhang, et al., 2019; Piparo, Scheib, Frei, Williamson, Grigorov,

& Chou, 2008; Williams et al., 2015). Furthermore, α-amylase and α-glucosidases have different protein structures as they belong to glycosyl hydrolase Families 13 and 31, respectively. α-Amylase as an endo-acting enzyme has the catalytic active site located in a wide and shallow groove on the protein surface, while α-glucosidases have narrow and deep catalytic pockets with an exo-acting mechanism (Bernardi, 2015; Henrissat & Davies, 1997). Therefore, the differences in the protein structures of starch digestive enzymes and the chemical ring structures of flavonoids can cause the different interactions between enzymes and flavonoids.

In this chapter, 14 flavonoids which have different ring structures were used to understand the structural specificity of flavonoids toward α-amylase and α-glucosidases, giving insight into the molecular requirement of flavonoids to selectively inhibit starch digestive enzymes.

4.3 Materials and methods

4.3.1 Materials

Myricetin, Gossyptin, Quercetagetin, Morin, Quercetin, Fisetin, 3',4'-dihydroxylflavonol, orobol, luteolin, kaempferol, galangin, eriodictyol, (+)-catechin, (-)-epicatechin were purchased from Sigma-Aldrich, MO, USA). Maltopentaose (Carbosynth, San Diego, USA) and maltose (Sigma-Aldrich, MO, USA) were used as substrates. Human pancreatic α-amylase was purchased from Lee Biosolutions (MO, USA). Glucose, maltotriose, and maltotetraose were also purchased from Sigma-Aldrich (MO, USA) to use as standards for HPAEC analysis.

4.3.2 Preparation of N-terminal MGAM

The purified N-terminal subunit of α-glucosidases (Nt-MGAM or maltase) was provided by Dr. David R. Rose's laboratory at University of Waterloo, Canada. Briefly, human Nt-MGAM was expressed in Drosophila S2 cells. Nickep-Sepharose resin was used to isolate the secreted proteins from the cell media that were further purified using anion exchange chromatography (Rossi et al., 2006; Sim et al., 2010)

4.3.3 Determination of protein concentration

Protein concentration of Nt-MGAM solution was determined using the BCA protein assay kit (Thermo Scientific, CA, USA). Protein solution (25 µL) was mixed with 200 µL of working reagent and then incubated at 37 °C for 30 min. Absorbance was read at 562 nm using a microplate reader (SpectraMAx 190, Molecular Devices, CA, USA). Bovine serum albumin was used as the standard in a range from 20 to 2000 ug/mL.

4.3.4 Measurement of enzyme activity

Nt-MGAM activity was determined by measuring the released glucose from maltose as substrate. The maltose solution (2.5%, w/v) was pre-warmed in a thermomixer at 37 °C and 800 rpm for 10 min. The pre-warmed substrate solution was mixed with Nt-MGAM solution and then incubated at 37 °C and 800 rpm for 15 min. The reaction solution was placed into a boiling water bath to terminate the enzyme reaction. The amount of released glucose was determined by the glucose oxidase/peroxidase (GOPOD) method. One unit (U) of Nt-MGAM was defined as the amount of enzyme that produces 1 mg of glucose released from maltose (2.5%, w/v) per 3 min at pH 6.9 and 37 °C.

4.3.5 *In vitro* inhibition assay

Flavonoid stock solutions were prepared in dimethyl sulfoxide (DMSO) within a range of concentrations from 10 to 100 µM. The flavonoid solutions were pre-warmed with substrates, maltopentaose (5%, w/v) for human pancreatic α-amylase and maltose (2.5%, w/v) for Nt-MGAM, in a thermomixer at 37 °C and 800 rpm for 10 min. The solutions were reacted with enzyme solutions, human pancreatic α-amylase () and Nt-MGAM (), at 37 °C and 800 rpm for 15 min, and then placed into boiling water to stop the enzyme reaction. The test solutions were further diluted 200 times with water and then filtered through a 0.45 µm nylon syringe filter prior to characterization of the digested products in the test solutions by HPAEC equipped with an electrochemical detector (Dionex, CA, USA). Filtered samples (25 µL) were injected into a CarboPac PA100 analytical column (4 x 250 mm), previously equilibrated with 120 mM sodium

acetate in 200 mM sodium hydroxide at a flow rate of 1 mL/min. Data analysis was conducted by Chromeleon 7 software (Dionex, CA, USA).

4.3.6 Statistical analysis

Differences across treatments were analyzed by one-way and two-way ANOVA with Tukey's multiple comparison test as the post-hoc test using SAS v.9.4 software (SAS Institute, Cary, NC, USA) to form statistical groupings. When *P* value was lower than 0.05, it was considered as a significant difference.

4.4 Results and discussions

4.4.1 Specific activity of Nt-MGAM

Specific activity of purified Nt-MGAM was measured with maltose as substrate by the GOPOD method and BCA protein assay kit (Table 1). Nt-MGAM showed 17.02 (U/ mg protein) of specific activity. Both subunits of MGAM share activities toward linear α-1, 4 linkages, but Nt-MGAM has the higher activity on shorter glucose oligomers, whereas the higher activity of Ct-MGAM is on longer glucose oligomer (Jones et al., 2011; Lin, Hamaker, & Nichols, 2012; Sim, Quezada-Calvillo, Sterchi, Nichois, & Rose, 2008). Therefore, Nt-MGAM has been ascribed as the maltase due to the higher hydrolysis property for maltose.

4.4.2 Profile of the digested products from substrates with flavonoids

The digested products from substrates by human pancreatic α-amylase (HPA) and Nt-MGAM with 100 μM of 14 flavonoids were determined using HPAEC (Figure 1 and 2). Firstly, maltose and maltotriose were mainly released from maltopentaose as the substrate by HPA. HPA has the catalytic active site between the second and third subsites from reducing end on five glucosyl substrate binding subsites, to maltose and maltotriose as the main digested products (Brayer, Luo, & Withers, 1995; Brayer et al., 2000). Gossyptin showed the highest inhibition (91.94%) of HPA among the 14 flavonoids. Quercetin (82.73%), luteolin (77.14%), orobol (70.6%), kaempferol (51.51%), myricetin (50.81%), and fisetin (50.4%) showed higher inhibition

property over 50% against HPA, while quercetagetin (38.03%), galangin (32.01%), morin (30.61%), 3',4'-dihydroxylflavonol (24.92%), eriodictyol (18.2%), (-)-epicatechin (7.48%), and (+)-catechin (5.68%) have lower inhibitory effects under 50%.

To screen the inhibition property of 14 flavonoids toward Nt-MGAM, maltose was used as substrate due to the higher ability of Nt-MGAM to digest shorter linear α-1, 4 linkage. Glucose from maltose was measured as the final digested product by the enzyme reaction. Interestingly, 14 flavonoids showed different inhibition properties against Nt-MGAM, compared to HPA. Quercetagetin (62.55%) has the highest inhibition activity for Nt-MGAM, followed by gossypetin (58.1%), fisetin (55.56%), 3',4'-dihydroxylflavonol (53.67%), quercetin (51.75%), myricetin (48.16%), orobol (43.15%), luteolin (28.17%), (-)-epicatechin (26.44%), eriodictyol (25.42%), (+)-catechin (16.76%), morin (11.41%), kaempferol (5.58%), galangin (4.72%).

The differences in the protein structure of HPA and Nt-MGAM, belonging to the glycosyl hydrolase Families 13 and 31 (GH13 and GH31) in the CAZy system, are responsible for the different inhibition of HPA and Nt-MGAM by 14 flavonoids used in this chapter (Henrissat et al., 1997).

4.4.3 Inhibition property of flavonoids against starch digestive enzymes

As shown in Figures 3 and 4, the different concentrations of flavonoids at 10, 50, and 100 μM were applied to test the inhibition property for HPA and Nt-MGAM. The inhibition properties of quercetin, gossypetin, luteolin, and orobol toward HPA were dramatically increased by increasing the concentrations. Meanwhile, eriodictyol, (+)-catechin, and (-)-epicatechin showed weaker or even negligible inhibition properties with increasing concentrations. Quercetin, gossypetin, and luteolin with higher inhibition properties could be considered as potential natural inhibitors to inhibit α-amylase activity.

The overall inhibition property of flavonoids toward Nt-MGAM was lower than the inhibition of HPA, showing 62.55% of the maximum inhibition of Nt-MGAM by quercetagetin. Quercetin, myricetin, 3',4'-dihydroxylflavonol, fisetin, quercetagetin, gossypetin, and orobol showed the increasement of inhibition property through the concentration rage, while galagin, kaempferol, morin, (+)-catechin, and (-)-epicatechin exhibited slight or even no increase of inhibition property for Nt-MGAM.

Quercetin, myricetin, gossypetin, fisetin, and orobol have the higher inhibition property for both of enzymes, HPA and Nt-MGAM. Lutelin, galangin, and kaempferol showed higher selectivity toward HPA, whereas 3',4'-dihydroxylflavonol, quercetagetin, and (-)-epicatechin selectively inhibited Nt-MGAM. The different selectivity of flavonoids could be applied to control glucogenesis with slower, but more complete, digestion of starch in the small intestine for improved glycemic response and minimized adverse side effects (Simsek et al., 2015).

4.4.4 Structural specificity of flavonoids to inhibit starch digestive enzymes

As shown in Figure 5, the 14 flavonoids used in this study have different inhibition properties against HPA and Nt-MGAM based on their chemical ring structures. Luteolin and eriodictyol have similar chemical structure, except for the double bond between C2 and C3, and had different inhibition properties, 77.14% and 18.20% by luteolin and eriodictyol, toward HPA. Furthermore, (+)-catechin and (-)-epicatechin, which do not have the double bond, showed the lowest inhibition activities of under 10%. It means the double bond between C2 and C3 plays a critical role to inhibit HPA (Lim, Zhang, et al., 2019; Piparo et al., 2008). The double bond can increase the planarity of flavonoids and then make the π-π interaction between flavonoids and HPA which has the catalytic active site located in a wide and shallow groove on the protein surface. Quercetin has an additional hydroxyl group at C3 compared to luteolin. Quercetin and luteolin showed 51.75% and 28.17% of the inhibition for Nt-MGAM. Additionally, luteolin showed a similar inhibition for Nt-MGAM with eriodictyol, which has the identical structure except the double bond. Based on the inhibition activities from these three flavonoids, it is likely that the hydroxyl group at C3 is intimately related to inhibition of Nt-MGAM activity, whereas the double bond has a minor effect on its inhibition. Terminal A and B ring structures on flavonoids have different effects on the inhibition of HPA and Nt-MGAM. The terminal A and B rings are equally important to lay parallel on the substrate binding sites of HPA to form π-staking interaction as fisetin, kaempferol, 3',4'-dihydroxylflavonol, and galangin, which have differences in A and B ring structures, showed similar inhibition properties. Meanwhile, the four flavonoids showed different inhibition patterns against Nt-MGAM. Fisetin and 3',4'-dihydroxylflavonol have a higher inhibition than kaempferol and galangin. It seems likely that terminal B ring structure is more important to inhibit Nt-MGAM, than A ring structure to direct the B ring down into the catalytic active site located in a narrow and

deep pocket in the protein structure. Quercetin with the hydroxyl group at B3 has a higher inhibition property toward HPA and Nt-MGAM compared to kaempferol and morin which have different structures of the hydroxyl group at B3. The hydroxyl group at B3 on the flavonoid structure is particularly important to inhibit both of HPA and Nt-MGAM. Additionally, the hydroxyl group at A5 is also important for the inhibition of HPA. The hydroxyl groups at B3 and A5 with the double bond could be considered as key structures to lay flavonoids parallel on the catalytic active site located in the wide and shallow groove on the protein surface, leading the π-π interaction between flavonoids and HPA (Williams et al., 2015).

4.4.5 Correlation of the molecular weight and number of hydroxyl groups of flavonoids with their inhibition property

Although it is believed that the high molecular weight and number of hydroxyl groups of flavonoids are intimately related to their interaction with protein (Bordenave, Hamaker, & Ferruzzi, 2013), the inhibition of starch digestive enzymes has no correlations with the molecular weight and number of hydroxyl groups on flavonoid structures, leading to low correlation coefficients (Figure 6). It is likely that the inhibition property of flavonoids could be derived from the location of hydroxyl groups on flavonoid ring structure (Xiao, Kai, Yamamoto, & Chen, 2013; Xiao, Ni, Kai, & Chen, 2013), instead of the molecular weight and number of hydroxyl groups. The planarity of flavonoids also plays an important role in their inhibition of starch digestive enzymes (Piparo et al., 2008).

4.5 Conclusions

Flavonoids as natural inhibitors could be considered as an approach for controlling starch digestion rate by inhibiting enzyme activities to manage diets related disease such as type 2 diabetes and obesity. In this study, 14 different flavonoids, which have different chemical ring structures such as the number and location of hydroxyl groups, the double bond between C2 and C3, *trans* and *cis* configurations, and isomerism, were used to understand the structural requirements of flavonoids to inhibit starch digestive enzymes. All flavonoids showed different inhibition properties against HPA and Nt-MGAM based on their chemical ring structures. Quercetin and gossypetin showed the highest inhibition property for both HPA and Nt-MGAM

among the tested flavonoids. They could be used as strong natural inhibitors. Myricetin and fisetin inhibited both HPA and Nt-MGAM with about 50% of inhibition, whereas luteolin and 3',4'-dihydroxylflavonol showed differential selectivity against HPA and Nt-MGAM. Luteolin has the higher specificity toward α-amylase with 77.14% of the inhibition, whereas 3',4'-dihydrxoylflavonol showed the higher selectivity for Nt-MGAM with 53.67%. The difference in chemical structures of both flavonoids is responsible for the higher selectivity toward α-amylase and the α-glucosidases. The higher selectivity could be applied to selectively inhibit starch digestive enzymes to control glucogenesis with slow but complete digestion without the adverse side effects such as blotting and diarrhea that occur with pharmaceutical starch-degrading enzyme inhibitors. Myricetin, gossypetin, and quercetagetin have the same number of hydroxyl groups on the chemical ring structures, while they showed different inhibition properties against HPA and Nt-MGAM. Moreover, quercetin showed higher inhibition of starch digestive enzymes compared to morin which has a shifted hydroxyl group from B3 on to B2. It means that the inhibition property of flavonoids is strongly related with certain specific chemical structures, rather than higher number of hydroxyl groups and the molecular weight as has been proposed by others. The different configuration of flavonoids is also important to inhibit starch digestive enzymes. Quercetin, (+)-catechin, and (-)-epicatechin have different configurations such as planar, and *cis* and *trans.* Particularly, the planar configuration has more potential to inhibit starch digestive enzymes as quercetin with the planar configuration has higher inhibition property compared to (+)-catechin and (-)-epicatechin. The double bond between C2 and C3 is intimately related to the inhibition of α-amylase. Flavonoids with the double bond have a higher degree of planarity, forming the π-π interaction between flavonoids and α-amylase. In addition, the hydroxyl group at C3 on the flavonoid chemical structure plays a critical role to inhibit Nt-MGAM, and potentially the other α-glucosidases, as the inhibition property of quercetin with the hydroxyl group at C3 is higher than luteolin without the hydroxyl group. The hydroxyl groups at A5 and B3 with the double bond between C2 and C3 are the essential structural requirements for inhibiting α-amylase activity. The structure can lay flavonoids parallel on the cleavage site located in the wide and shallow groove on the protein surface, thus forming the π-staking interaction between flavonoids and the catalytic residues. Meanwhile, the hydroxyl groups of B3 and C3 are particularly important to inhibit Nt-MGAM and α-glucosidases by binding with the catalytic active sites positioned at the narrow and deep pockets in the protein structure.

This study would help in the future development of functional foods or natural inhibitors to control starch digestion rate to prevent diet related diseases by improving glycemic response without adverse side effects.

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Table 4-1. Specific activity of purified Nt-MGAM

	Nt-MGAM
Specific activity (U/ mg protein)	17.02 ± 1.41

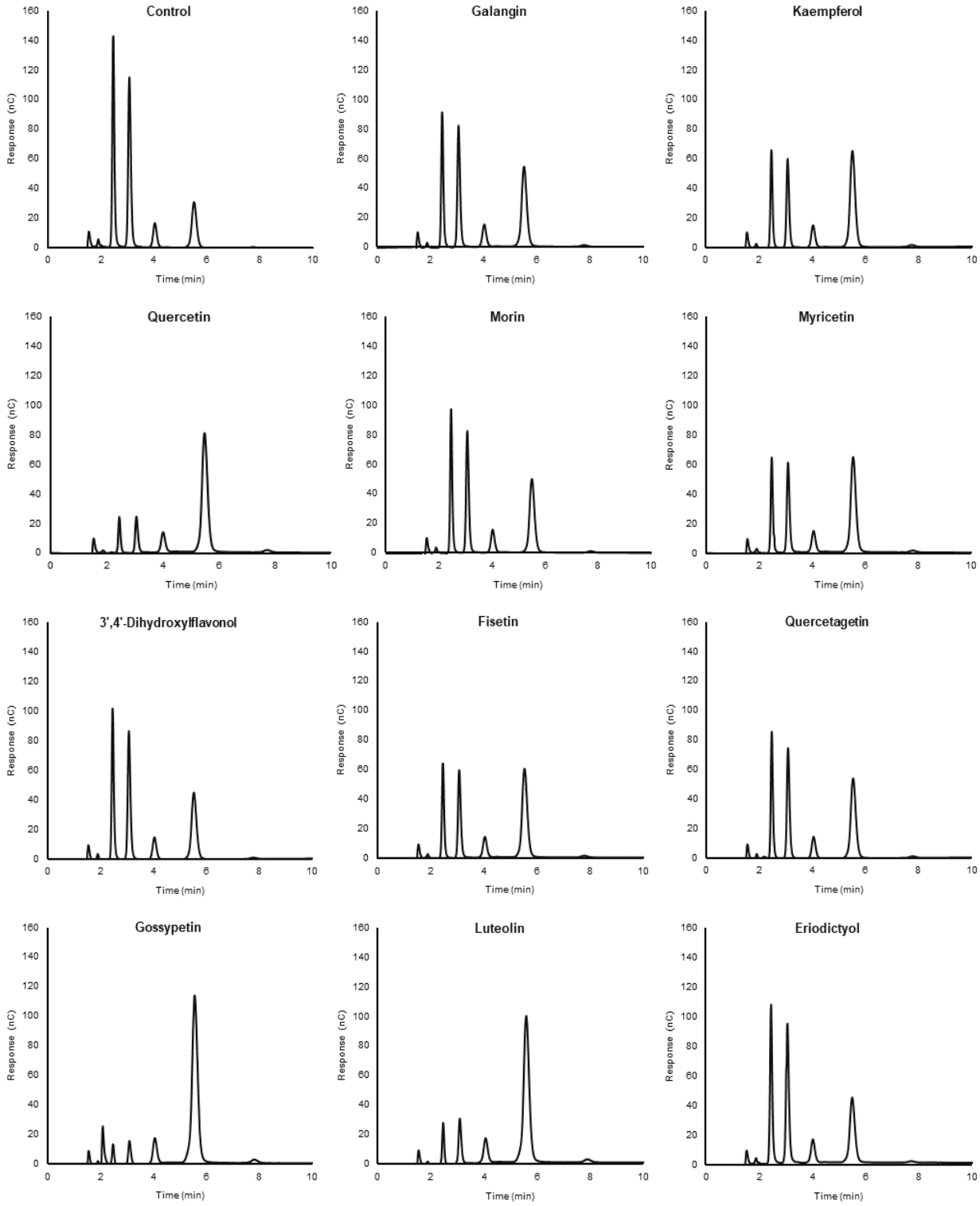

Figure 4-1. Profile of digested products from maltopentaose by HPA with flavonoids

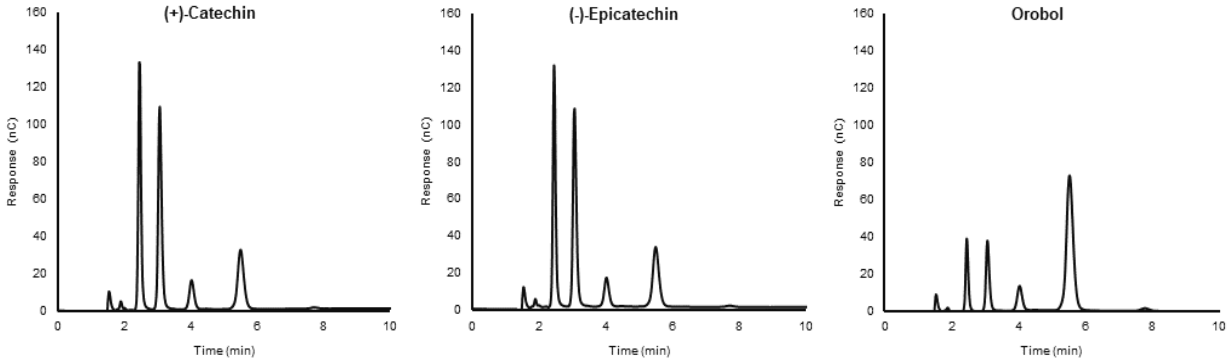

Figure 4-2 (continued). Profile of digested products from maltopentaose by HPA with flavonoids

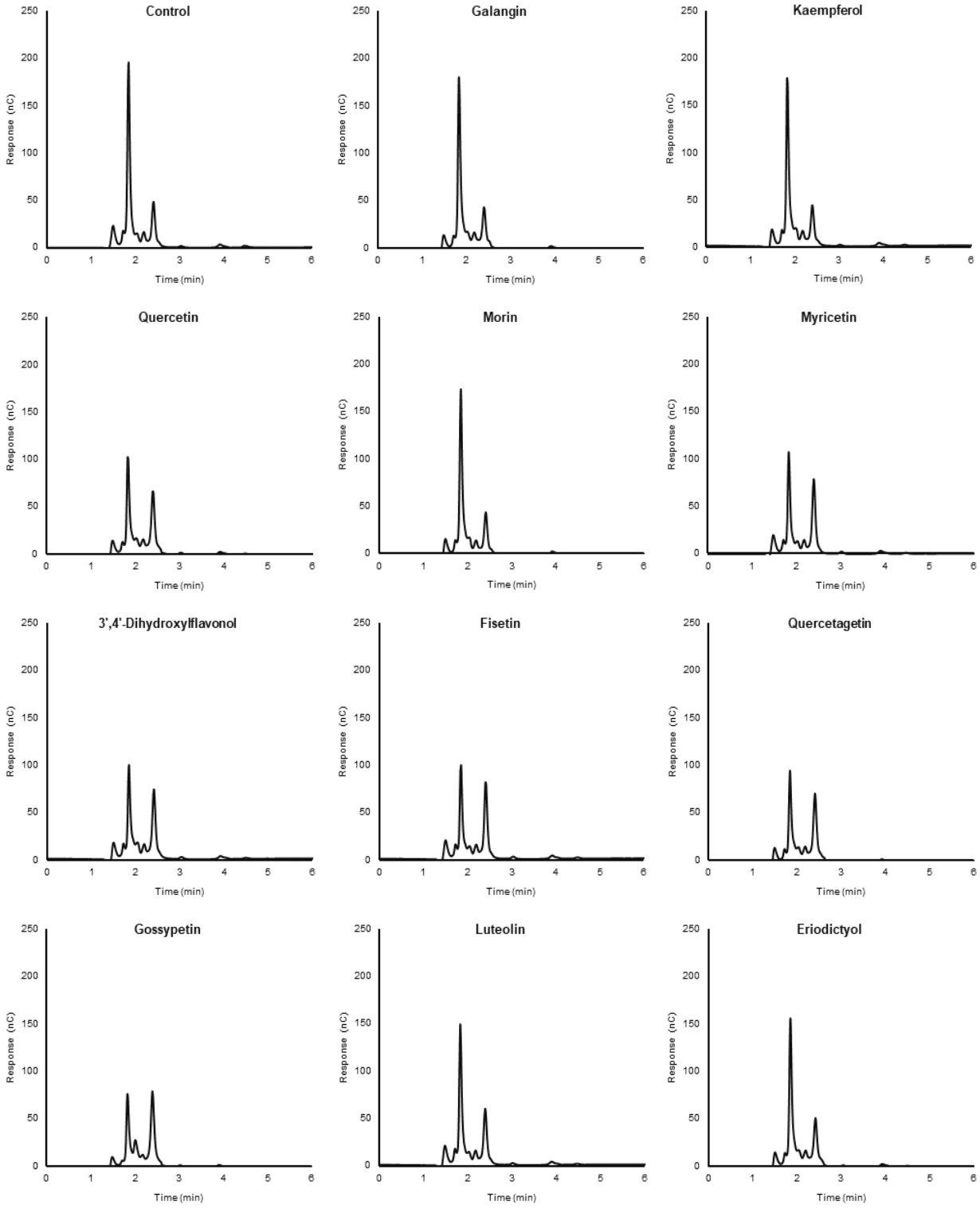

Figure 4-3. Profile of digested product from maltose by Nt-MGAM with flavonoids

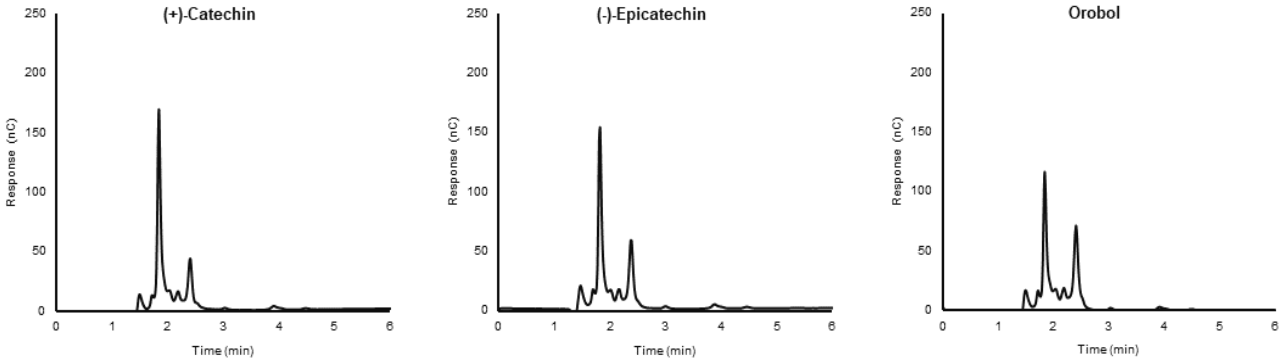

Figure 4-4 (continued). Profile of digested product from maltose by Nt-MGAM with flavonoids

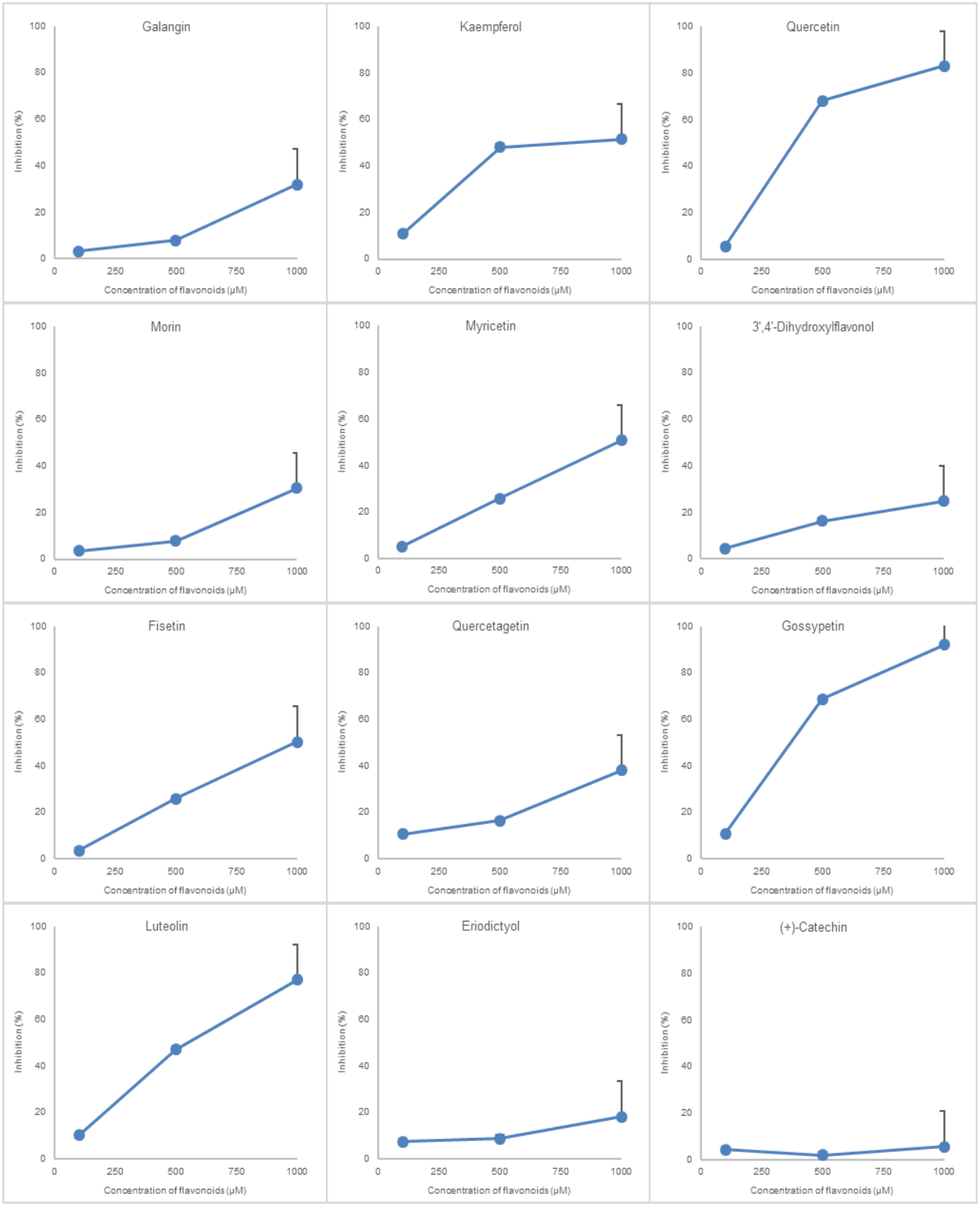

Figure 4-5. Inhibition property of flavonoids against α-amylase

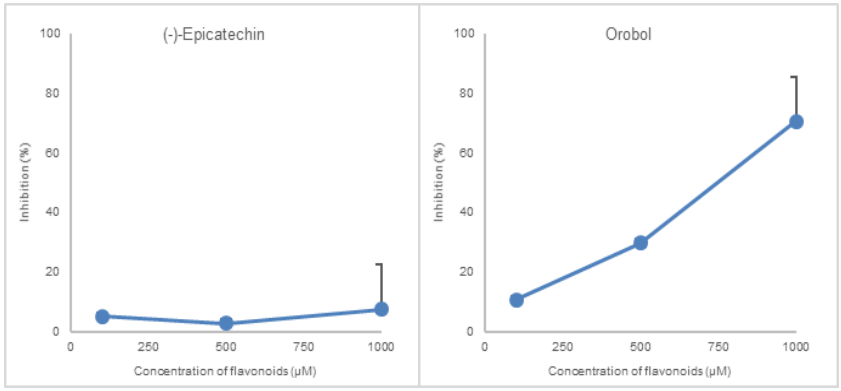

Figure 4-6 (continued). Inhibition property of flavonoids against α-amylase

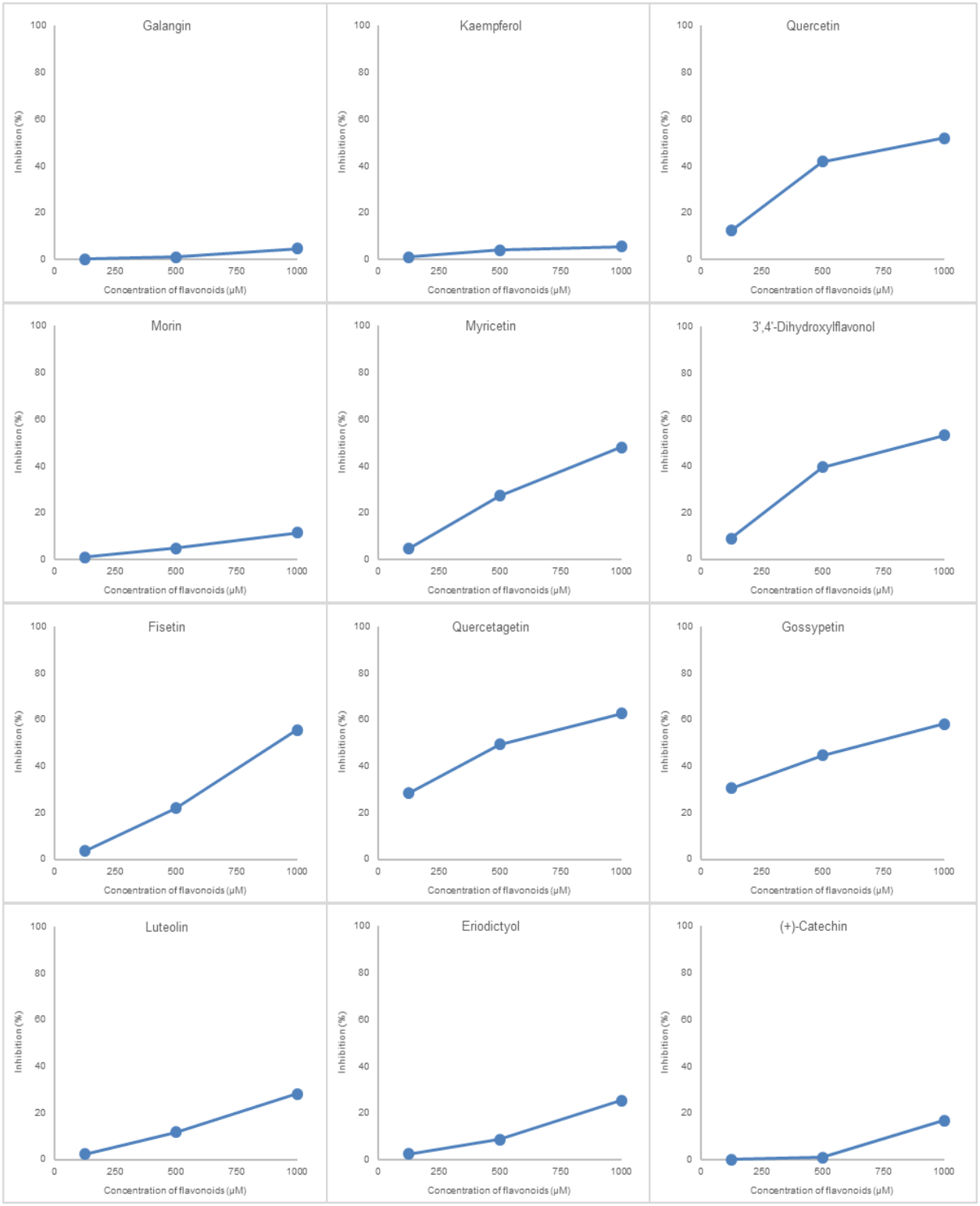

Figure 4-7. Inhibition property of flavonoids against Nt-MGAM

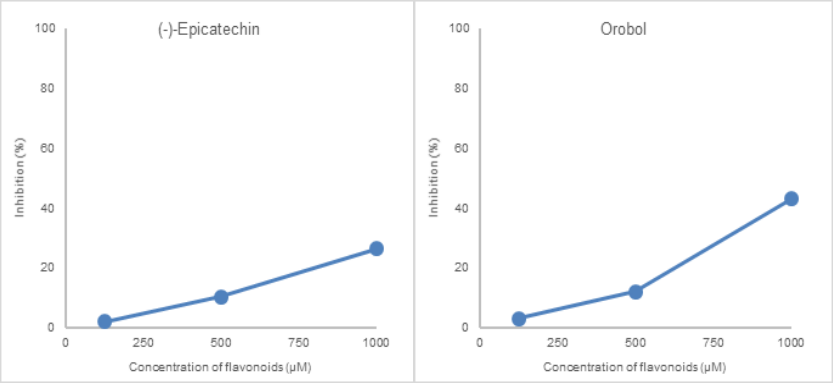

Figure 4-8 (continued). Inhibition property of flavonoids against Nt-MGAM

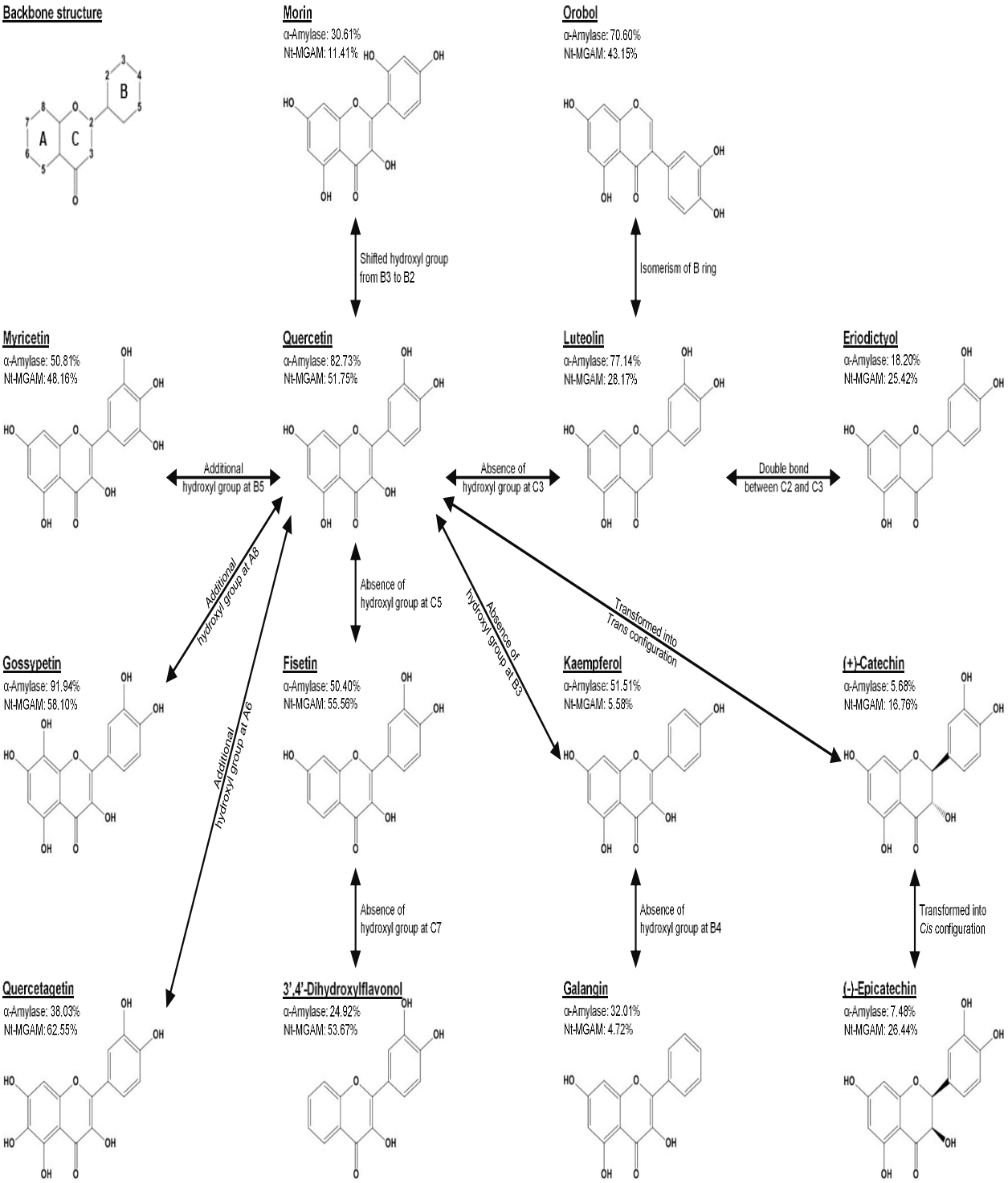

Figure 4-9. Structural specificity of flavonoids to inhibit starch digestive enzymes

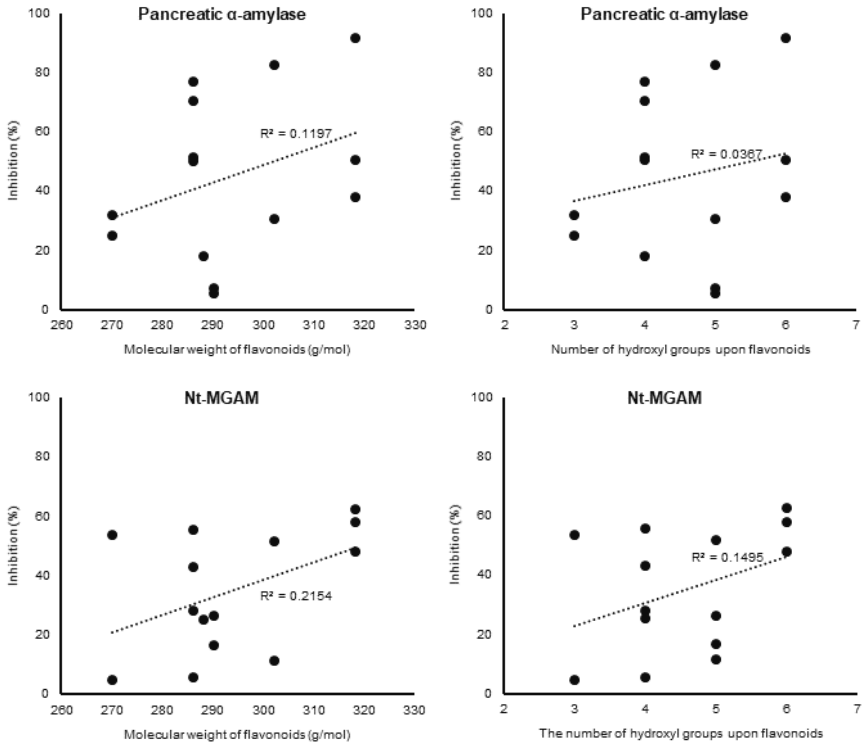

Figure 4-10. Correlation of the molecular weight and number of hydroxyl groups of flavonoids with their inhibition property

CHAPTER 5. SELECTIVE INHIBITION OF STARCH DIGESTIVE ENZYMES TO CONTROL STARCH DIGESTION RATE AND MODERATE GLYCEMIC RESPONSE

5.1 Abstract

Luteolin and 3',4'-dihydroxylflavonol have different inhibition properties and binding affinities toward starch digestive enzymes depending on their chemical ring structures. The terminal hydroxyl groups in the A ring with the double bond between C2 and C3 on luteolin were previously found to play a critical role in the inhibition of α-amylase, leading to the π-π interaction between the flavonoid and α-amylase. On the other hand, the hydroxyl group at C3 is particularly important in the inhibition of the α-glucosidases. In this study, luteolin and 3',4'-dihydroxylflavonol were used to selectively inhibit starch digestive enzymes to control starch digestion rate and modulate glycemic response, thereby minimizing adverse effects of high postprandial glycemia. The effects of specific inhibition of α-amylase and α-glucosidases were evaluated using a mice model. Selective inhibition of α-amylase using luteolin showed slow and sustainable glycemic response after the oral gavage of starch with luteolin. Furthermore, the α-amylase inhibition transferred more starch portion into the end of small intestine, resulting in triggering of the secretion of the GLP-1 hormone which can regulate food intake and appetite.

5.2 Introduction

Over 400 million people worldwide suffer from diabetes and it is closely associated with obesity (Mathers & Loncar, 2006). In part, it has been believed that the diet-related diseases could be managed by controlling starch digestion rate in the gastrointestinal tract (Lee, Bello-Pérez, Lin, Kim, & Hamaker, 2013). Starch is first digested by α-amylases from the salivary gland and pancreas and then the digested products are further converted into glucose by the mucosal α-glucosidases in the small intestine. The glucose is transferred to the blood stream by the glucose transporters located at the apical surface of enterocyte (Zhang, Ao, & Hamaker, 2006; Zhang & Hamaker, 2009). Antidiabetic drugs such as acarbose miglitol, and voglibose have been used to control starch digestion rate by inhibiting enzyme activities (Chiasson, Josse, Gomis, Hanefeld, Karasik, & Laakso, 2002; Chiasson et al., 2003). Unfortunately, the use of the drugs as inhibitors often cause adverse side effects such as abdominal pain, flatulence, and diarrhea due to dumping of the undigested starch fraction into the colon. The substantial restriction of starch digestion originates from poor specificity which leads to severe inhibition or even shutdown of enzyme activities (Williams et al., 2015). Therefore, selective and less severe inhibition of starch digestive enzymes could be considered as an approach to control starch digestion rate with minimizing the side effects (Lee et al., 2012; Simsek, Quezada-Calvillo, Ferruzzi, Nichols, & Hamaker, 2015).

Flavonoids have received significant attention to use as natural inhibitors of starch-degrading enzymes with partial inhibition property (Williamson, 2013). Previous studies have shown a selective inhibition property of natural phenolic compounds against starch digestive enzymes (Kim, Kwon, & Son, 2000; Tadera, Minami, Takamatsu, & Matsuoka, 2006). Based on chemical ring structure, flavonoids selectively inhibit starch digestive enzymes. As shown from work in Chapter 4 of this thesis, the double bond between C2 and C3 on the flavonoid ring structure plays an important role to inhibit α-amylase, leading to the π-π interaction between flavonoids and α-amylase, while the hydroxyl group at C3 is particularly important to inhibit α-glucosidases (Bernardi, 2015; Lim, Zhang, Ferruzzi, & Hamaker, 2019; Piparo, Scheib, Frei, Williamson, Grigorov, & Chou, 2008; Williams et al., 2015). Additionally, the OH group present in position B3 is required to bind to both starch digestive enzymes. The hydroxyl group at A5 is essential to interact with α-amylase (Xiao, Kai, Yamamoto, & Chen, 2013; Xiao, Ni, Kai, & Chen, 2013). The selectivity of phenolic compounds to different digestive

enzymes is likely the result of their different protein structures, as α-amylase and the α-glucosidases belong to different glycosyl hydrolase Families 13 and 31 (Henrissat & Davies, 1997). It is hypothesized here that the selective inhibition of starch digestive enzymes by flavonoids could be applied to control glucogenesis with slow, but still complete, digestion rate in the small intestine to improve the postprandial glycemic response. To do this, an understanding is needed of the contribution of each enzyme inhibition to increase the selective inhibition efficiency. Selective inhibition targeted at one single starch enzyme could be considered as a key approach to control the starch digestion rate, diminishing the specific problems that result from currently available starch digestive enzyme inhibitors.

In Chapter 4, the structural specificity of 14 flavonoids was evaluated and they showed different specificity toward α-amylase and the α-glucosidases depending on their chemical ring structures. Among the flavonoids, luteolin had higher selectivity for human pancreatic α-amylase with 77.2% inhibition property, while 3',4'-dihydroxylflavonol showed the higher selectivity toward the α-glucosidases, represented by Nt-MGAM, with 53. 7% inhibition. Therefore, in this present study, they were used as specific enzyme inhibitors to understand the contribution of each enzyme inhibition to control starch digestion rate, compared to quercetin which showed the relatively high inhibition of both α-amylase and α-glucosidase. The finding obtained from this study may provide insights into how to apply flavonoids as natural inhibitors and the future development of functional foods or pharmaceutical drugs for controlling starch digestion rate.

5.3 Materials and methods

5.3.1 Materials

Quercetin, 3',4'-dihydroxylflavonol, and luteolin were purchased from (Sigma-Aldrich, MO, USA). Maltopentaose (Carbosynth, San Diego, USA) and maltose (Sigma-Aldrich, MO, USA) were used as substrates. Human pancreatic α-amylase was purchased from Lee Biosolutions (MO, USA). Glucose, maltotriose, and maltotetraose were also purchased from Sigma-Aldrich (MO, USA) to use as standards for HPAEC analysis. All buffer chemicals and reagents were obtained from Sigma-Aldrich (IL, USA).

5.3.2 Preparation of MGAM

Two purified terminal subunits of α-glucosidases, N-terminal maltase-glucoamylase (Nt-MGAM) and C-terminal maltase-glucoamylase (Ct-MGAM), were provided by Dr. David R. Rose's laboratory at University of Waterloo, Canada. Briefly, Ct-MGAM from mouse was generated by recombinant expression in baculovirous Sf9 insect cell systems (Jones et al, 2011, Lee, JBC toggling). Nt-MGAM from human was expressed in Drosophila S2 cells. The expressed recombinant proteins were further purified using nickel-nitrilotriacetic acid affinity column chromatography with imidazole gradient from 10 to 250 mM (Rossi et al., 2006; Sim et al., 2010, Lee plosone).

5.3.3 Determination of protein concentration

Protein concentration of MGAM solutions was determined using the BCA protein assay kit (Thermo Scientific, CA, USA). Protein solution (25 µL) was mixed with 200 µL of working reagent and then incubated at 37 °C for 30 min. Absorbance was read at 562 nm using a microplate reader (SpectraMAx 190, Molecular Devices, CA, USA). Bovine serum albumin was used as the standard in a range from 20 to 2000 ug/mL.

5.3.4 *In vitro* inhibition assay

Flavonoid stock solutions were prepared in dimethyl sulfoxide (DMSO) within a range of concentrations from 10 to 100 µM. The flavonoid solutions were pre-warmed with substrates, maltopentaose (1 mM, K_m = 0.94 mM) for human pancreatic α-amylase and maltose (2 mM, K_m = 2.29 mM for Nt-MGAM and 1.65 mM for Ct-MGAM) for the two subunits of MGAM, in a thermomixer at 37 °C and pH 6.9 for 10 min. The solutions were reacted with the enzyme solutions, human pancreatic α-amylase (1 µg) and each subunit of MGAM (1 µg), at 37 °C and pH 6.9 for 15 min, and then placed into boiling water to terminate the enzyme reaction. The test solutions were further diluted 50 times with water and then filtered through a 0.45 µm nylon syringe filter prior to characterization of the digested products in the test solutions using HPAEC (Dionex, CA, USA) equipped with an electrochemical detector. Filtered samples (25 µL) were injected into a CarboPac PA-100 analytical column (4 x 250 mm, Dionex, CA, USA), previously equilibrated

with 120 mM sodium acetate in 200 mM sodium hydroxide at a flow rate of 1 mL/min. Data analysis was conducted by Chromeleon 7 software (Dionex, CA, USA) and the inhibition property of flavonoids was calculated as a percentage of the digested products of the control.

5.3.5 Binding affinity determination

Fluorescence intensity of starch digestive enzymes with flavonoids was recorded on a fluorescence spectrophotometer (Agilent, CA, USA). Starch digestive enzymes (1 µg) were mixed with flavonoids (10, 50 and 100 µM) and then the mixtures were incubated at 37 °C and pH 6.9 for 15 min. Emission spectra of the test solutions was scanned in the wavelength range of 320 to 550 nm at 295 nm of excitation wavelength. Both excitation and emission slit widths were set as 10 nm. Data analysis was carried out using Cary Eclipse software (Agilent, CA, USA).

5.3.6 Animal acute study

Ten week old male C57BL/6J mice (The Jackson Laboratory, ME, USA) were maintained under controlled temperature at 21 °C, humidity at 55%, and 12 hour light/dark cycle with standard chow diet and water provided *ad libitum*. All animal treatments were approved by the Purdue Animal Care and Use Committee (PACUC). Starch gelatinized with water (2 g/kg body weight), maltose (2 g/kg body weight), and glucose (2 g/kg body weight) were orally administered with the different concentrations (0.8 g/kg body weight) of luteolin, quercetin, and 3',4'-dihydroxylflavonol following a 16 h overnight fasting. The oral administration volume of solution was 10 mL/kg body weight, and the concentration of DMSO to dissolve flavonoids was 10% in the test solution. After oral administration, blood samples were obtained from tail vein at 15, 30, 60, and 120 min to measure the glucose level in blood using a glucose monitor (Bayer, IN, USA). Mice were euthanized and then cervical dislocation was conducted to collect blood via cardiac puncture, pancreas, small intestine, and luminal contents from the proximal and distal parts of the small intestine at 15, 30, 60, and 120 min after the oral gavage of gelatinized starch (2 g/kg body weight) with the different concentrations (0.1, 0.4, and 0.8 g/kg body weight) of flavonoids. The level of total GLP-1 in blood plasma was determined using enzyme-linked immunosorbent assay (ELISA) kits (EZGLP1T-36K, MilliporeSigma, MA, USA) according to the manufacturer's instructions. Pancreas and small intestine were homogenized with sodium phosphate buffer (100

mM and pH 6.9). The homogenates were centrifuged at 4 °C and 12,000 rpm for 10 min and then the supernatants were collected for use as enzyme solutions. The inhibition property of luteolin, quercetin, and 3',4'-dihydroxylflavonol against the enzyme solutions extracted from pancreas and small intestine was evaluated using the HPAEC method (Lim et al., 2019). The freeze-dried luminal contents from the small intestine were dissolved in DMSO and centrifuged at room temperature and 8,000 rpm for 20 min. The supernatant was further mixed with 80% ethanol and then centrifuged at room temperature and 8,000 rpm for 20 min to precipitate starch followed by vacuum drying. The molecular size and debranched chain length distribution of starch extracted from the luminal contents were analyzed using HPSEC (Agilent, CA, USA), and HPAEC (Dionex, CA, USA) systems. Starch dissolved in DMSO was mixed with 80% ethanol. The mixed solution was centrifuged at room temperature and 8,000 rpm for 15 min and the residue was further dried using a vacuum dryer (Fisher Scientific, NH, USA). Water at 60 °C was added to the dried starch sample and filtered with a 5 µm nylon syringe filter. The filtered sample solution (100 µL) was injected into tandem column system with Superdex 200 and 30 columns (GE Healthcare, NJ, USA) to analyze the molecular size distribution of starch extracted from the lumen contents. Pure water with 0.02% sodium azide was used as mobile phase at a flow rate of 0.4 mL/min. In the experiment to evaluate the debranched chain length distribution of starch obtained from the small intestine, the vacuum-dried starch was treated with pullulanase (0.7 units, Megazyme, IL, USA) and isoamylase (0.1 units, Megazyme, IL, USA) in sodium acetate buffer (100 mM and pH 5.0) at 40 °C for 48 h. The enzymatic treated solution (25 µL) was applied to a CarboPac PA-100 analytical column (4 x 250 mm, Dionex, CA, USA) following the 0.22 µm nylon membrane filtration. The sample solution was eluted with 150 mM sodium hydroxide (eluent A) and 600 mM sodium acetate in 120 mM sodium hydroxide (eluent B) at a flow rate of 1 mL/min. Data was analyzed by Chromeleon 7 software (Dionex, CA, USA) and the chain length distribution was calculated as a percentage of the total peak area.

5.3.7 Statistical analysis

Data analysis was conducted by one way and two way ANOVA with Tukey as the post-hoc test using SAS v.9.4 software (SAS Institute, Cary, NC, USA) to form statistical groupings. When the *P* value was lower than 0.05, it was considered as significant difference.

5.4 Results and discussion

5.4.1 Profile of substrates degradation by starch digestive enzymes with flavonoids

As shown Figure 1, luteolin, and 3',4'-dihydroxylflavonol had different inhibition patterns against human pancreatic α-amylase and two subunits of α-glucosidases, while quercetin inhibited both enzyme activities. In pancreatic α-amylase inhibition, luteolin and quercetin released similar amounts of digested products, mainly maltose and maltotriose, from maltopentaose as substrate, thus showing approximately equal and substantive inhibition effect. 3',4'-Dihydroxylflavonol showed a much higher amount of the digested products, compared to luteolin and quercetin, indicating poor inhibition of α-amylase. Contrarily, luteolin and 3',4'-dihydroxylflavonol had opposite inhibition activities toward the α-glucosidases, Ct- and Nt-MGAM. 3',4'-Dihydroxylflavonol showed a similar amount of released glucose from maltose by the two subunits of α-glucosidases with quercetin. The HPAEC chromatogram of luteolin showed a higher amount of glucose, indicating its poor inhibition effect. Therefore, luteolin is selective in inhibition towards α-amylase and 3',4'-dihydroxylflavonol for the α-glucosidases, and quercetin has inhibition effect on both types of enzymes. The different selectivity of flavonoids could be utilized to selectively inhibit starch digestive enzymes to control starch digestion rate, minimizing the adverse side effects observed by the strong pharmaceutical grade inhibitors.

5.4.2 Inhibition property of flavonoids at different concentrations

The three flavonoids at the different concentrations (10, 50, and 100 μM) were applied to inhibit starch digestive enzymes. Even though all three flavonoids showed differences at 50 μM to inhibit human pancreatic α-amylase, luteolin and quercetin had a similar higher inhibition at 100 μM with 77.14% and 82.73%, respectively. 3',4'-Dihydroxylflavanol had the lower inhibition property (24.92%). At 100 μM, the three flavonoids fell in different statistical groupings. Quercetin and 3',4'-dihydroxylflavonol had were both inhibitory of the α-glucosidases and were in the same statistical group for two subunits of MGAM at 50 and 100 μM. Luteolin was significantly less inhibitory toward Ct- and Nt-MGAM. It is important to note that quercetin has the inhibition property for both enzymes, whereas luteolin and 3',4'-dihydroxylflavonol showed the different selectivity to inhibit human pancreatic α-amylase and the two subunits of MGAM. The different structures of enzymes and flavonoids are responsible for the selectivity of inhibition of enzymes by flavonoids.

5.4.3 Time-dependent inhibition property of flavonoids

Figure 5-3 shows the time-dependent inhibition property of flavonoids. All three flavonoids at 100 μM had no time-dependent inactivation of starch digestive enzymes. The level of inhibition activity of flavonoids remained constant for over 2 h. Again, luteolin showed the higher selectivity for human pancreatic α-amylase, while 3',4'-dihydroxylflavonol selectively inhibited Ct- and Nt-MGAM. The absence of the hydroxyl group at C3 on the luteolin structure is associated the lower inhibition of mucosal α-glucosidases, whereas the absence of the hydroxyl groups at the terminal A ring on 3',4'-dihydroxylflavonol relates to its lower selectivity for human pancreatic α-amylase. Furthermore, the higher inhibition of quercetin against α-amylase and the α-glucosidases is likely to be caused by the presence of double bind and the OH positioned at C3 and A5, forming the π-π interaction with human pancreatic α-amylase and binding by B ring specific entry into the narrow and deep catalytic pocket of the mucosal α-glucosidases (Davies & Henrissat, 1995; Henrissat et al., 1997).

5.4.4 Inhibition of starch digestive enzyme at the different concentrations by flavonoids

Different concentrations of starch digestive enzymes were applied to determine the inhibition property of flavonoids. All three flavonoids showed increase in reaction velocity with increasing enzyme amounts (Figure 5-4). Luteolin and quercetin slowed down the reaction velocity of pancreatic α-amylase, while 3',4'-dihydroxylflavonol and quercetin decelerated the reaction of the MGAM subunits of the α-glucosidases. Furthermore, the results showed that all straight lines passed through the origin, indicating reversible activity.

5.4.5 Fluorescent quenching of starch digestive enzymes with flavonoids

Figure 5-5 shows the fluorescence spectra of starch digestive enzymes with flavonoids at 100 μM. Luteolin and quercetin decreased the emission spectra of human pancreatic α-amylase, compared to the addition of 3',4'-dihydroxylflavonol. On the other hand, 3',4'-dihydroxylflavonol and quercetin dropped the peak intensity of the two subunits of α-glucosidases, Ct- and Nt-MGAM. The different binding affinity between the flavonoids and enzyme proteins were likely the result of different ring structural features of flavonoids. The hydroxyl group at A5 on luteolin and quercetin should lead to binding within the wide and shallow groove on the α-amylase surface, while the hydroxyl group at

C3 on 3',4'-dihydroxylflavonol and quercetin is particularly important to enter the narrow and deep catalytic pocket in the α-glucosidases. Moreover, the different binding affinity of flavonoids toward starch digestive enzymes is intimately related to their selective inhibition of α-amylase and α-glucosidase.

5.4.6 Fluorescence intensity peaks of starch digestive enzymes at the different concentrations of flavonoids

Fluorescence quenching of starch digestive enzymes with the different concentrations of flavonoids was conducted to measure the emission spectra (Figure 5-6). All emission spectra were decreased by decreasing the flavonoids concentration. Luteolin and 3',4'-dihydroxylflavonol showed different binding affinities against α-amylase and α-glucosidases. Quercetin interacted with both enzymes. Therefore, quercetin can be considered as natural inhibitor to inhibit both of enzymes, while luteolin and 3',4'-dihydroxylflavonol can be used as specific inhibitors to selectively inhibit α-amylase or the α-glucosidases, respectively.

5.4.7 Postprandial glycemic response with flavonoids

Using the mice model, glycemic response was evaluated by the oral administration of digestible carbohydrates with flavonoids (Figure 5-7). Postprandial blood glucose level at 20 min was significantly lower for the oral administration of starch with luteolin and quercetin compared to control and 3',4'-dihydroxylflavonol. Interestingly, the stronger α-amylase inhibitors, luteolin and quercetin, treatments had a significantly higher blood glucose level at 60 min, indicating that luteolin and quercetin not only slow down starch digestion rate, but appear to extend digestion in the gastrointestinal tract. 3',4'-Dihydroxylflavonol and quercetin treatments significantly reduced the blood glucose level at 20 min after the oral gavage of maltose, which by-passes α-amylase and targets the α-glucosidases. While there was a reduction in peak blood glucose, there was not an extended glucose release as seen with inhibition of α-amylase. When mice were treated with glucose and flavonoids, to target inhibition at the glucose transporter level, all three flavonoids had no significant effect on postprandial glycemic responses.

Thus, quercetin reduced the blood glucose levels from the administration of both starch and maltose, corresponding with inhibition at both α-amylase and α-glucosidase levels, while luteolin

and 3',4'-dihydroxylflavonol showed reduction of postprandial glycemic response at α-amylase and α-glucosidase levels, respectively. Importantly, α-amylase inhibition had more contribution to improve the postprandial glycemic response with the reduced blood glucose level. Therefore, the selective inhibition of α-amylase could be considered as a key approach to control starch digestion rate and extend glucose release to obtain certain health benefits.

5.4.8 Level of GLP-1 after the oral administration of starch with flavonoids

Figure 5-8 shows the levels of total GLP-1 after the oral administration of starch with flavonoids, targeting the inhibition of α-amylase as described above. Luteolin and quercetin treatments resulted in substantially higher levels of GLP-1 at 60 and 120 min, compared to control (starch alone) and 3',4'-dihydroxylflavonol treatments. It is likely the result of inhibition of α-amylase causing the delivering of more starch portion into the distal small intestine where a greater number of the enteroendocrine L-cells exist, which secrete GLP-1. GLP-1 is one of the gut hormones active in appetite control (ref), and it means that the selective inhibition of α-amylase by flavonoids (i.e. luteolin and quercetin), with resultant triggering of the release of GLP-1, conceivably could be applied to control food intake to prevent, reduce, or even treat diet-related chronic diseases such as diabetes and obesity.

5.4.9 Inhibition of extracts from pancreas and small intestine by flavonoids

The inhibition property of flavonoids against extracts from pancreas and small intestine was determined (Figure 5-9). Luteolin and 3',4'-dihydroxylflavonol showed the expected different inhibition properties toward the extracts. Luteolin had the higher inhibition activity for the pancreatic extract, while 3',4'-dihydroxylflavonol showed the specific inhibition of extracts from the small intestine representing the α-glucosidases. Quercetin inhibited both extracts with the highest inhibition activities among three flavonoids. This finding lends support to the belief that luteolin selectively inhibits α-amylase and the selective inhibition of α-amylase could be considered as a key approach to control starch digestion rate for improved glycemic response, minimizing adverse side effects, and triggering GLP-1 secretion which can regulate food intake and appetite.

5.4.10 Molecular weight distribution of the digesta from the small intestine

As shown in Figure 5-10, the molecular weight distributions of the digested starch products from the small intestine was evaluated using HPSEC. Luteolin and quercetin treatments had the bigger molecular weight distribution during the 2 h digestion process in mice small intestine, compared to the control and 3',4'-dihydroxylflavonol treatments. This corroborates inhibition of α-amylase by flavonoids to slow down the starch digestion rate. Even though 3','4-dihydroxylflavonoid showed the bigger molecular weight distribution than control without flavonoids, the starch degraded molecules were smaller than those of the luteolin or quercetin treatments. It means that the selective inhibition of α-amylase has more contribution to modulate the digestion rate than α-glucosidases inhibition. Therefore, selective inhibition targeted at α-amylase could be applied to control glucose release with slow, but more complete, digestion rate, minimizing the side effects.

5.4.11 Chain length distribution of the digesta from small intestine

Figure 5-11 shows the debranched chain length distributions, representing remaining starch and starch degradation products, of the digesta from flavonoids treatments in the small intestine. All three flavonoids treatments showed the differences in short chain lengths (A + B1 chains) on the starch molecule. Luteolin and quercetin treatments had small amounts of chains from DP 1 to DP 9, compared to the control and 3',4'-dihydroxylflavonol treatments, while the digesta from luteolin and quercetin treatments contained higher amounts of chains from DP 10 to DP 25. It means that the digesta from luteolin and quercetin treatments were less digested having longer chain distributions in the remaining small intestine luminal starch, indicating again that α-amylase inhibition has more contribution to slow down starch digestion rate.

5.5 Conclusions

Luteolin and 3',4'-dihydroxylflavonol were used as enzyme specific inhibitors to selectively inhibit the two major starch digestive enzymes, α-amylase and α-glucosidases, compared to quercetin which had the higher inhibition property against both α-amylase and α-glucosidases. Luteolin showed selective inhibition activity for α-amylase, while 3',4'-dihydroxylflavonol

selectively inhibited α-glucosidases. Furthermore, the two flavonoids have the different binding affinity toward α-amylase and α-glucosidases. It is likely the result of the different chemical ring structure of luteolin and 3',4'-dihydroxylflavonol. Luteolin has terminal hydroxyl groups on its A ring. It is speculated that terminal hydroxyl groups with the double-bond between C2 and C3 on the luteolin ring structure forms the π-π interaction at the catalytic active site located in the wide and shallow groove on α-amylase surface. Meanwhile, 3',4'-dihydroxylflavonol has the hydroxyl group at C3 and the OH group is particularly important to inhibit α-glucosidases, leading to the postulate of the B ring specific entry into the narrow and deep catalytic pocket (Henrissat et al., 1997; Lim et al., 2019; Williams et al., 2015).

The specific inhibition of starch digestive enzymes was evaluated using a mice model. The *in vitro*-determined selective inhibition of α-amylase by luteolin showed sustainable and slow postprandial glycemic response with reduced blood glucose peak and extended high glucose response, compared to the α-glucosidase specific inhibition. It means that the control of starch digestion rate by the selective inhibition of α-amylase likely stimulates the gut-brain axis from the distal small intestine.

The use of pharmaceutical drugs to control starch digestion rate often results in the adverse side effects such as bloating, diarrhea, and abdominal pain due to dumping of a large portion of undigested starch portion into the colon (Chiasson et al., 2002). The severe restriction of starch digestion is derived from poorer specificity of the overly strong inhibitors against both α-amylase and α-glucosidases, resulting in substantial inhibition or even shutting down all enzyme activities. Therefore, the selective inhibition of α-amylase could be considered as a key approach to control glucogenesis with slow, but still complete, digestion rate, minimizing the side effects (Lim, Kim, Shin, Hamaker, & Lee, 2019).

The consumption of foods with certain flavonoids which have a comparably high inhibition property of α-amylase may have practical treatment implications for improved postprandial glycemic response as a dietary approach. Furthermore, the findings obtained from this study provide better insights into the development of functional foods for controlling starch digestion rate (Lim, Kim, Shin, Hamaker, & Lee, 2019).

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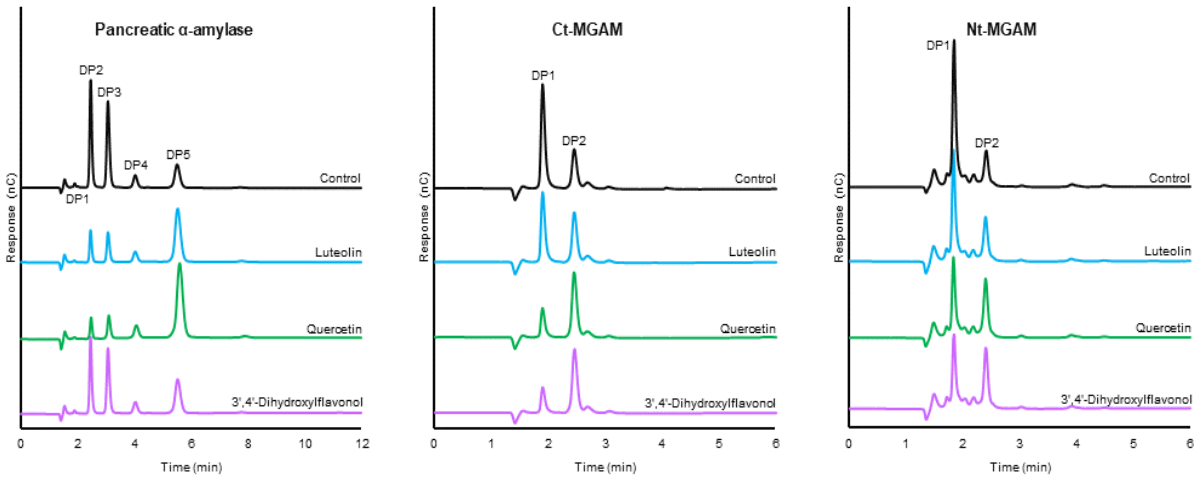

Figure 5-1. Profile of digested products by starch digestive enzymes with flavonoids

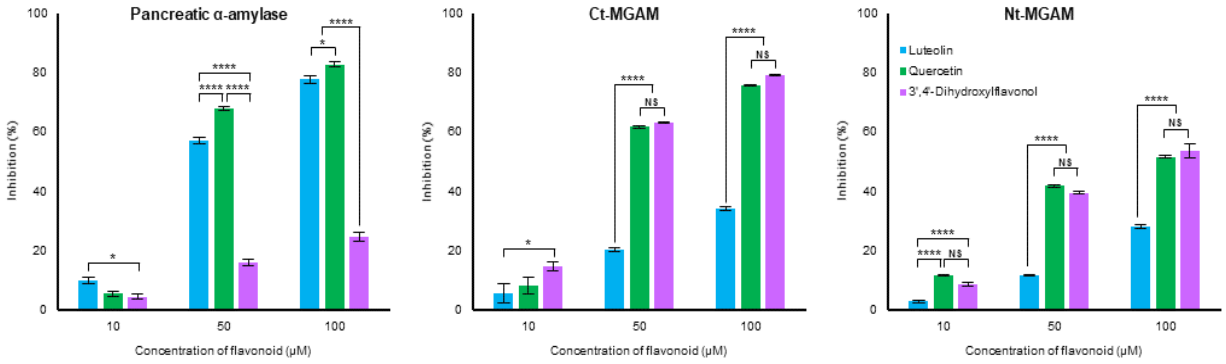

Figure 5-2. Effects of different concentration of flavonoids on the inhibition of starch enzymes

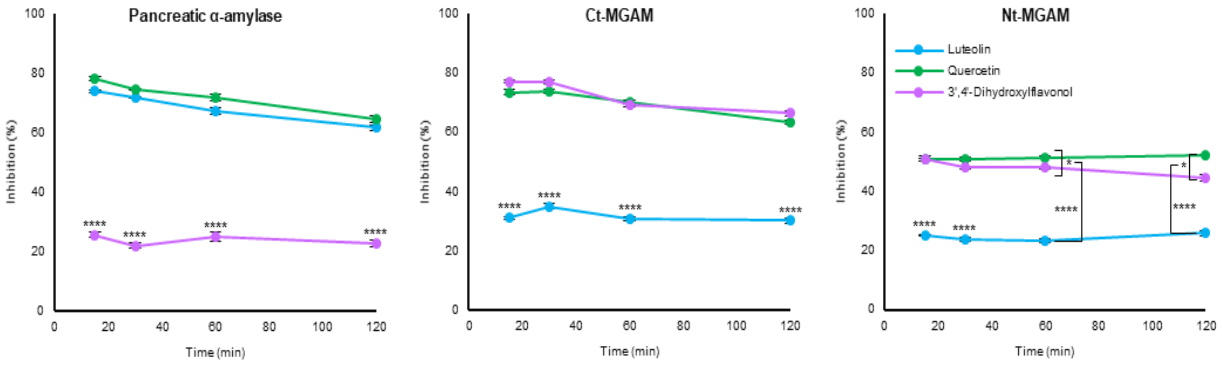

Figure 5-3. Time-dependent inhibition property of flavonoids towards starch digestive enzymes

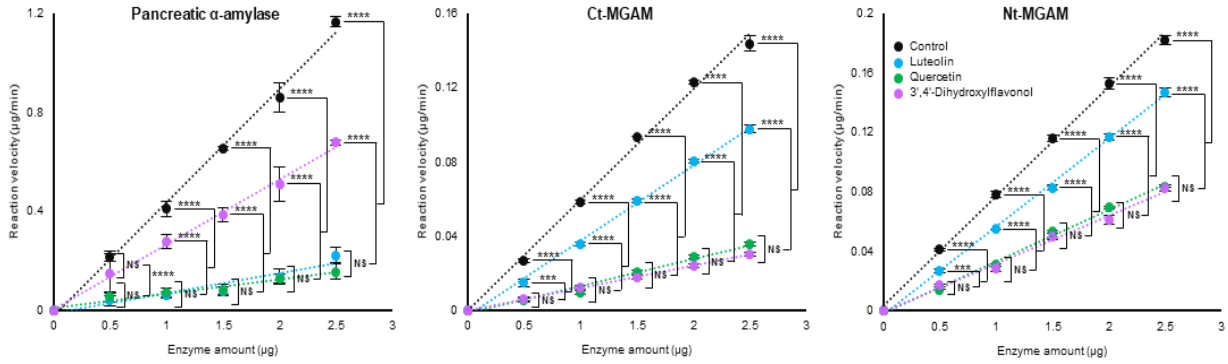

Figure 5-4. Inhibition of starch enzymes at the different concentrations by flavonoids

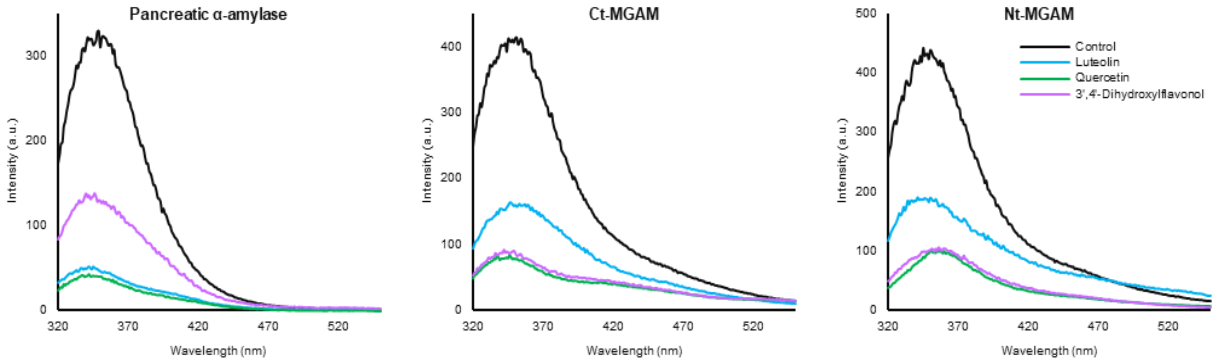

Figure 5-5. Fluorescence spectra of starch digestive enzymes with flavonoids

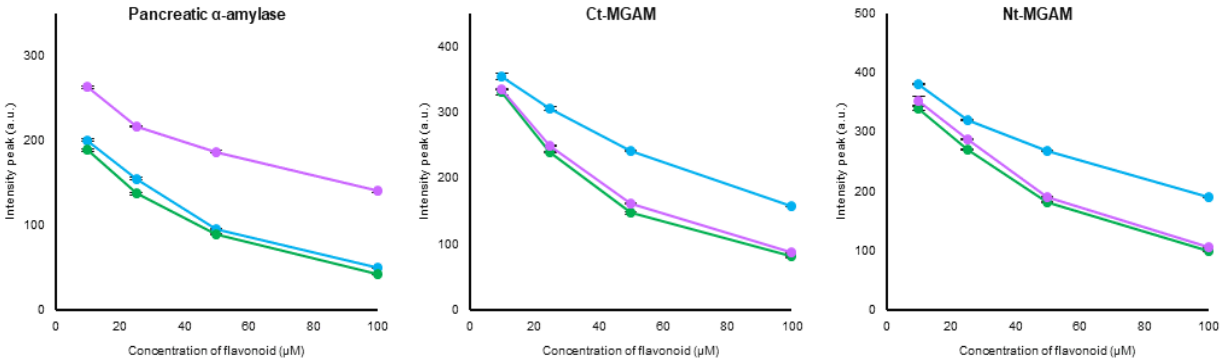

Figure 5-6. Fluorescence intensity peaks of starch enzymes at the different concentration of flavonoids

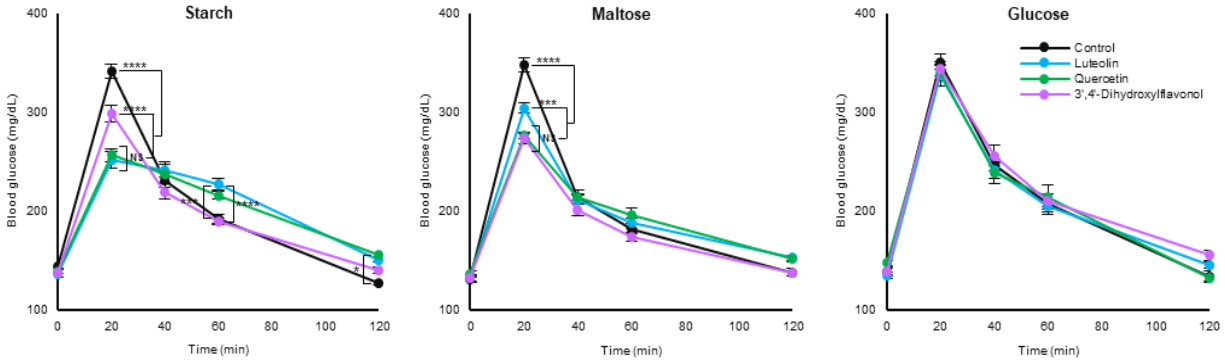

Figure 5-7. Glycemic response of digestible carbohydrates with flavonoids

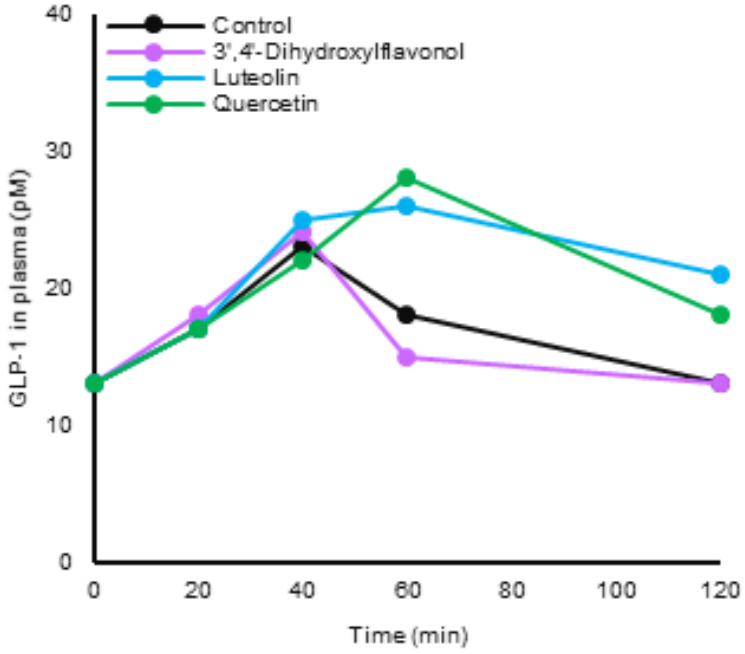

Figure 5-8. GLP-1 in plasma after oral administration of starch with flavonoids

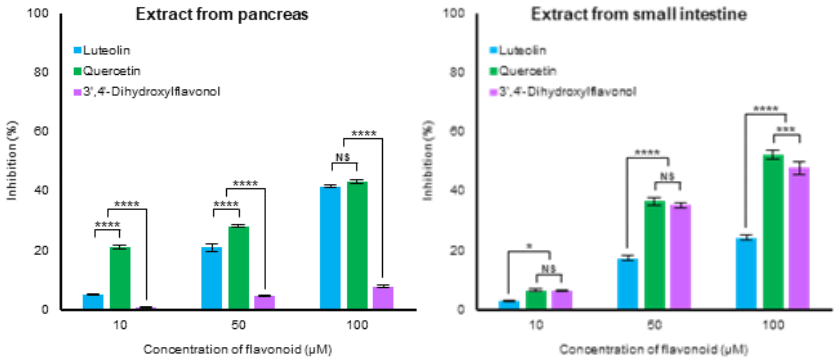

Figure 5-9. Inhibition of extracts from pancreas and small intestine by flavonoids

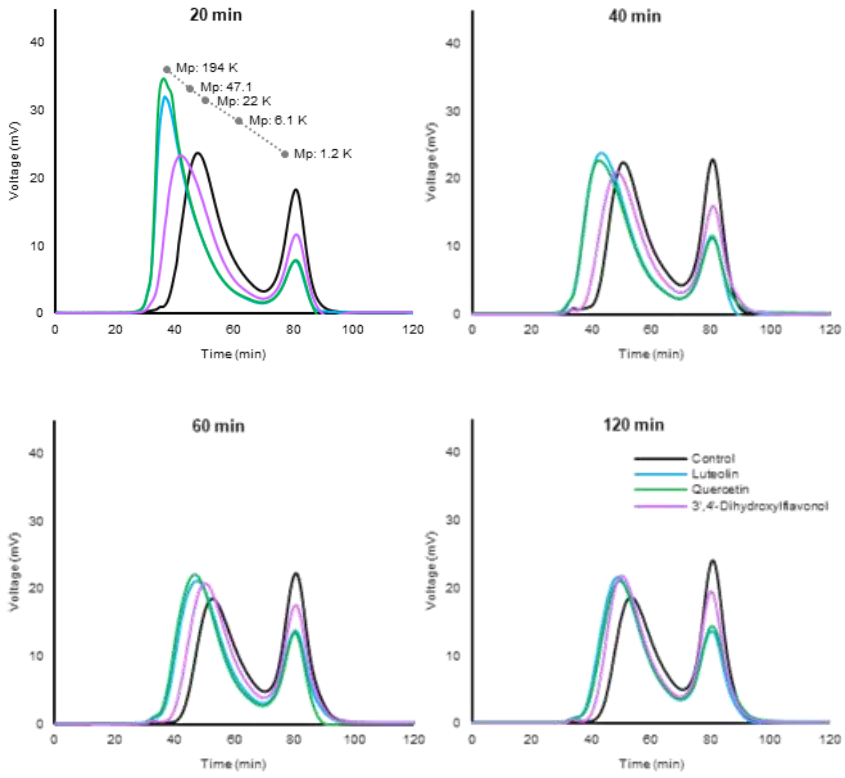

Figure 5-10. Molecular weight distribution of the digesta from the small intestine

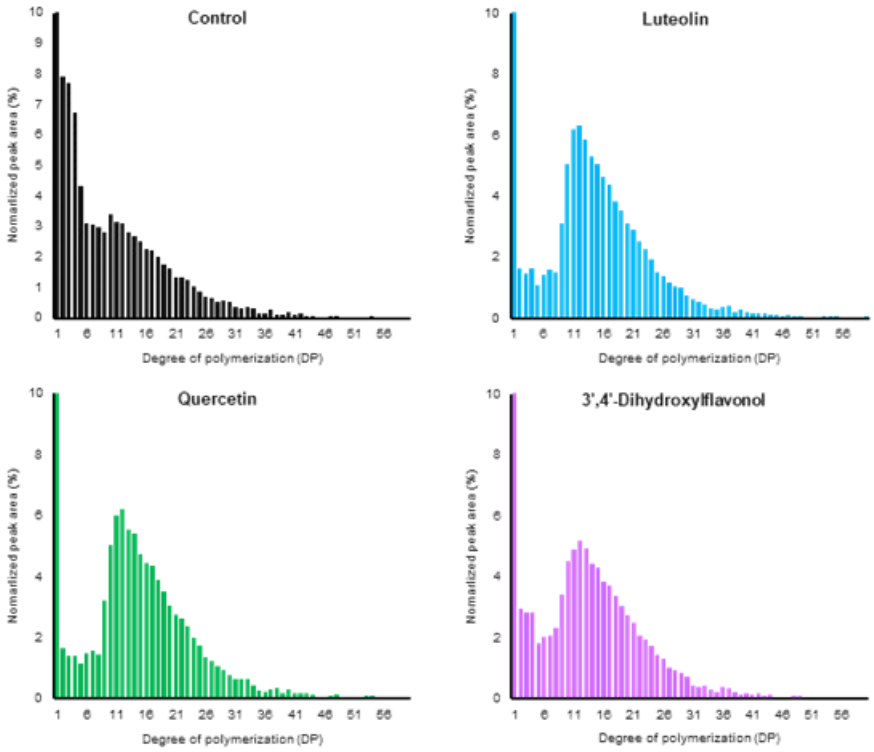

Figure 5-11. Chain length distribution of the digesta from the small intestine

CHAPTER 6. CONCLUSIONS AND FUTURE RECOMMENDATIONS

In this study, an accurate and precise HPAEC method was presented to evaluate the inhibition property of flavonoids against starch digestive enzymes, instead of the conventional colorimetric methods. Using a direct HPAEC measurement method, 14 flavonoids showed the structural specificity toward starch digestive enzymes. The double bond between C2 and C3 played a critical role to inhibit endo-acting α-amylase, leading to the π-π interaction, while the hydroxyl group at C3 was intimately related to the inhibition of exo-type α-glucosidases. The structural specificity of flavonoids for α-amylase and α-glucosidases is likely the result of different protein structures of the two starch digestive enzymes as they belong to glycosyl hydrolase Families GH13 and GH31, respectively. Furthermore, endo-acting α-amylase has catalytic active sites located in the wide and shallow groove on the protein surface, while exo-acting α-glucosidases has narrow and deep pockets as the catalytic active site. Among the 14 flavonoids, luteolin, quercetin, and 3',4'-dihydroxylflavonol were used to selectively inhibit starch digestive enzymes due to their higher selectivity against α-amylase and α-glucosidases. Luteolin showed the higher selectivity toward human pancreatic α-amylase, 3',4'-dihydroxylflavonol had selective inhibition for Nt-MGAM and Ct-MGAM of α-glucosidases, and quercetin showed the inhibition of both α-amylase and α-glucosidases. Based on animal acute study, it was noted that α-amylase inhibition has more contribution to control the starch digestion rate and glycemic response. Furthermore, more starch delivery into the end of small intestine can cause activation of the gut-brain axis, and perhaps ileal brake, by triggering the GLP-1 hormone which can regulate food intake and appetite. The selective inhibition of α-amylase can be considered as a key approach to control starch digestion rate, improving glycemic response, and activating the gut-brain axis and ileal brake, and minimizing adverse side effects such as bloating and diarrhea of pharmaceutical inhibitors.

To give insight into future work, there is a need to evaluate the inhibition property of conjugated phenolic compounds beyond aglycone flavonoids against starch digestive enzymes, as they are more common in nature. It is also needed to find certain natural compounds to inhibit two subunits of sucrase-isomaltase, because they are strongly responsible for hydrolysis of sucrose and branched α-1,6 linked glucans. In doing so, it would be possible to control the digestion rate of

sugar and digestible carbohydrate with a higher degree of α-1,6 linkages. Finally, a human study needs to be done to understand the effects of selective inhibition of α-amylase in the human body.

Knowledge gained in this study will help future developments of functional foods or pharmaceutical agents for controlling starch digestion rate and postprandial glycemia.

APPENDIX A. SUPPLEMENTAL INFORMATION FOR CHAPTER 3

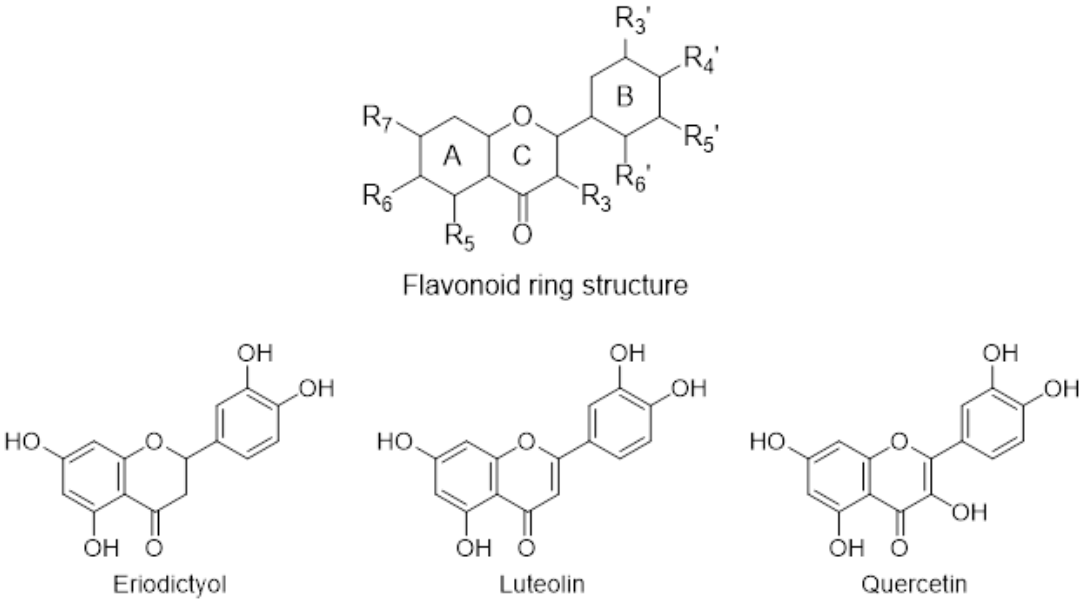

Figure A-1. Chemical structure of flavonoids used in chapter 3

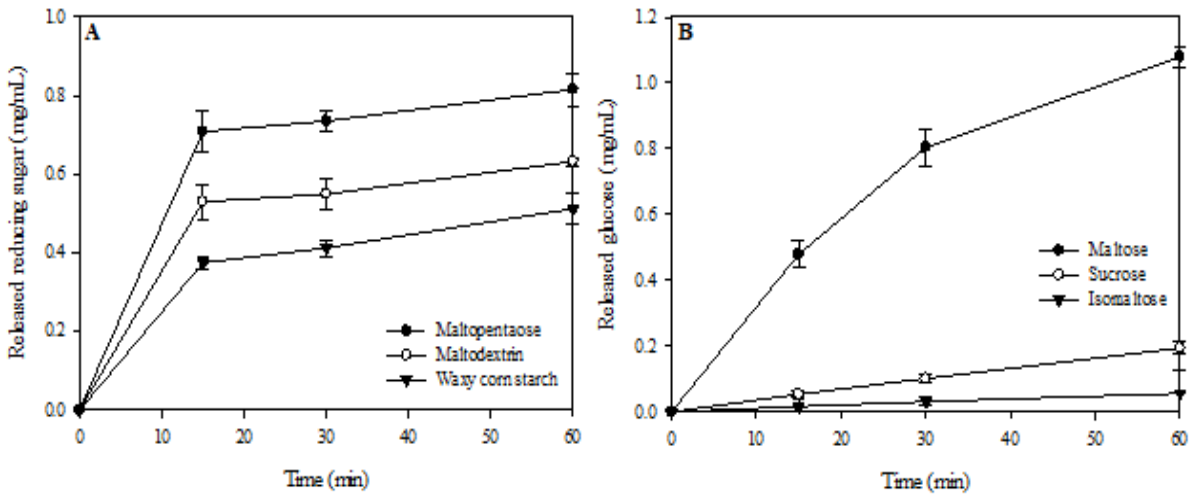

Figure A-2. Released digested products from different substrates by α-amylase (A) and α-glucosidases (B) measured by DNS and GOPOD methods

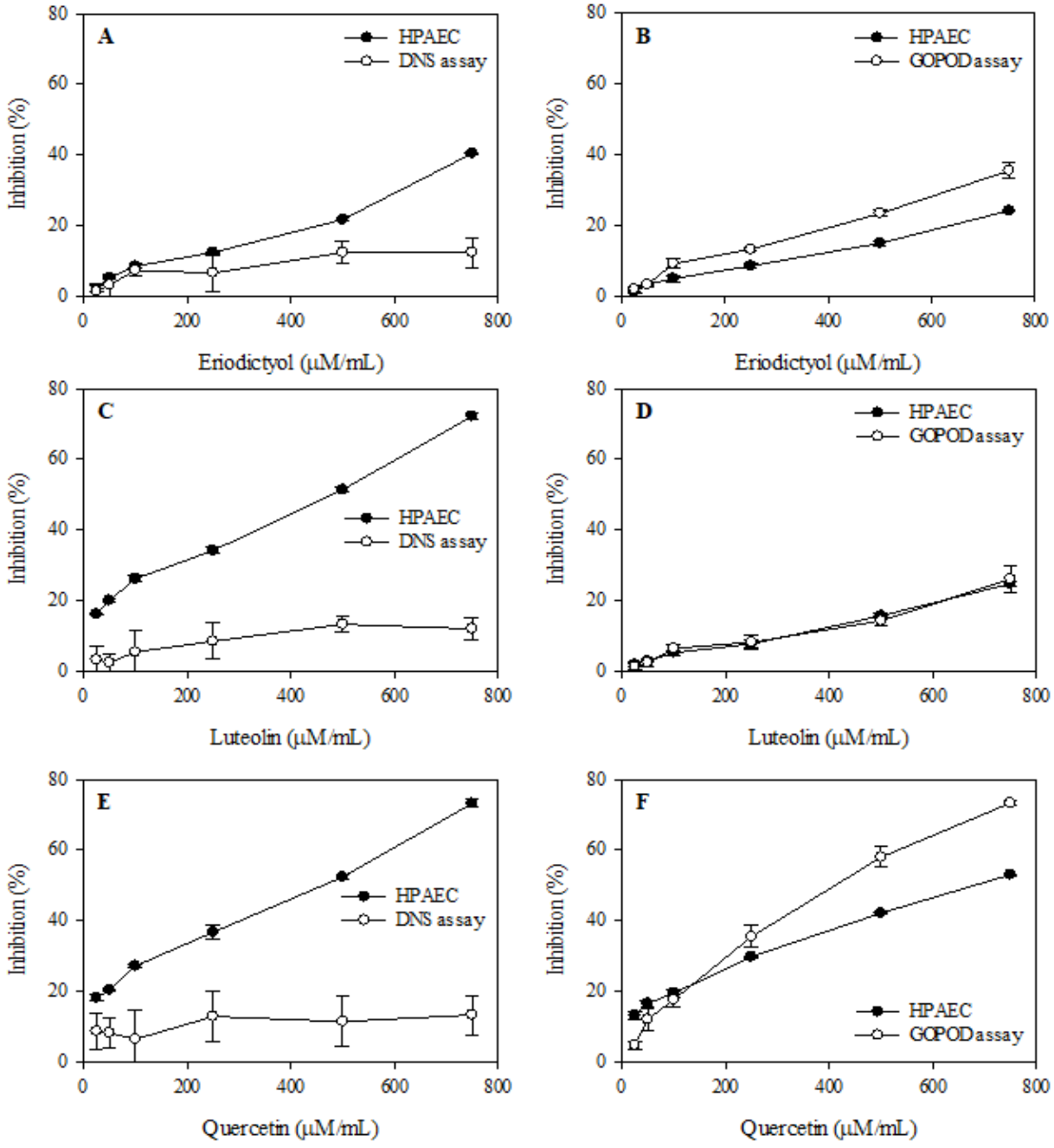

Figure A-3. Differences in inhibition activities (%) of flavonoids against α-amylase (A, C, and E) and α-glucosidases (B, D, and F) measured by colorimetric and HPAEC methods

APPENDIX B. SUPPLEMENTAL INFORMATION FOR CHAPTER 5

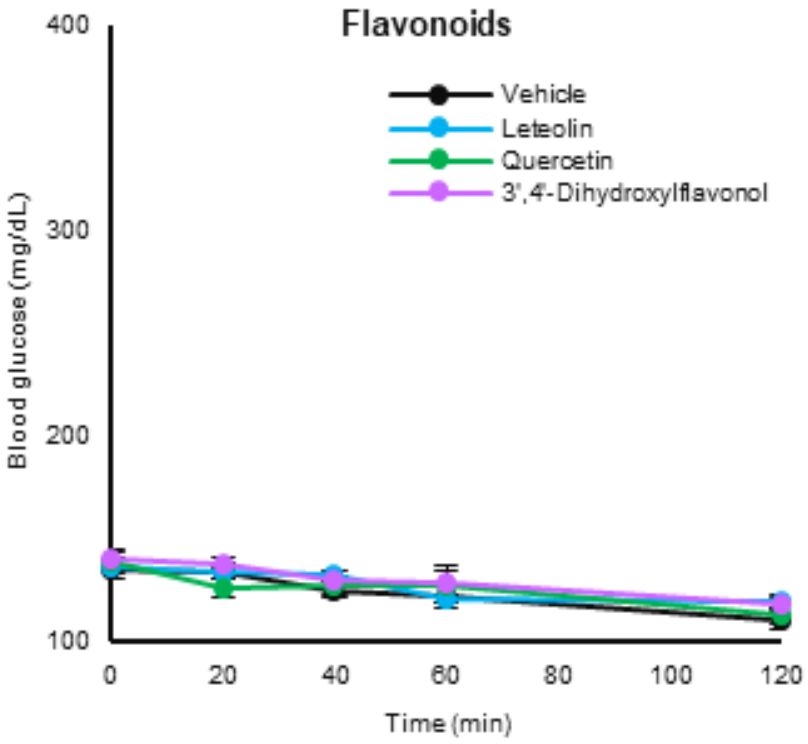

Figure B-1. Postprandial glycemic response after oral gavage of flavonoids

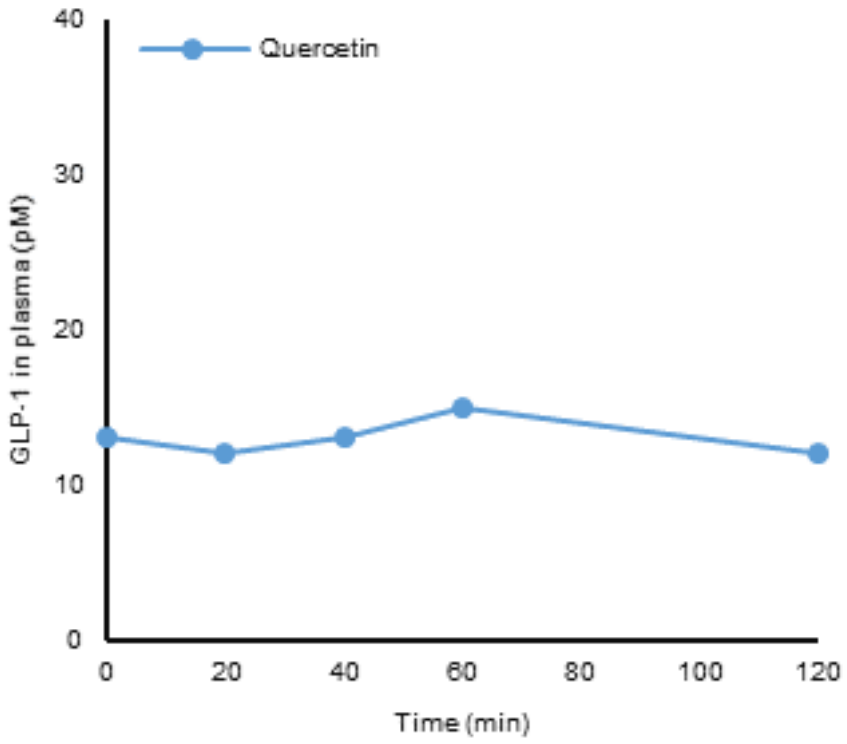

Figure B-2. The level of GLP-1 in plasma after oral gavage of quercetin

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