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INHIBITION OF ACTIVITIES OF INDIVIDUAL SUBUNITS OF INTESTINAL MALTASE-GLUCOAMYLASE AND SUCRASE-ISOMALTASE BY DIETARY PHENOLIC COMPOUNDS FOR MODULATING GLUCOSE RELEASE AND GENE RESPONSE

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MALTASE-GLUCOAMYLASE AND SUCRASE-ISOMALTASE BY DIETARY
PHENOLIC COMPOUNDS FOR MODULATING GLUCOSE RELEASE AND GENE
RESPONSE

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To my family

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ABSTRACT

Simsek, Meric. Ph.D., Purdue University, December 2014. Inhibition of activities of individual subunits of intestinal Maltase-Glucoamylase and Sucrase-Isomaltase by dietary phenolic compounds for modulating glucose release and gene response. Major Professor: Bruce R. Hamaker.

The occurrence of Type 2 diabetes is on the increase all over the world. Since free glucose is released through digestion of starch in the human diet, control of the starch digesting enzymes, particularly the intestinal mucosal α-glucosidases [Maltase-Glucoamylase (MGAM) and Sucrase-Isomaltase (SI)], has a potentially important role in both the etiology and treatment of this metabolic disorder. Inhibition of the activities of the intestinal α-glucosidases may be a promising way to moderate glucose delivery to the body. Although some commercial inhibitors, such as acarbose, have strong effect on these enzymes, and essentially block starch digestion, there is a need for new candidate inhibitors found in regular diets that still deliver glucose to the body in a slower way and have fewer side effects. Phenolics are known to have inhibitory effect on the intestinal α-glucosidases. For more precise control of glucose release in the small intestine, the concept of selective inhibition of the individual subunits (C terminal, Ct; N terminal, Nt) of MGAM and SI has been proposed. In this thesis work, we found that some phenolics selectively inhibit the individual mammalian recombinant subunits of MGAM and SI. For instance, chlorogenic acid and (-)-epigallocatechin gallate (EGCG) selectively inhibited the most active starch digesting subunit, Ct-MGAM (also called glucoamylase). We additionally used rat intestinal acetone powder and human intestinal tissue to investigate

the inhibitory effects of selected phenolics on α-glucosidases. Chlorogenic acid and EGCG showed the high inhibitory potency for maltase, sucrase and isomaltase activities of rat intestinal acetone powder. Also, chlorogenic acid notably inhibited the sucrase activity of human immunoprecipitated SI, while EGCG the maltase activity of human immunoprecipitated MGAM. Also explored, and for the first time, were the effects of some phenolic compounds on gene expression levels of MGAM and SI. The presence of phenolic compounds in mouse explants caused the generation of different molecular size forms of MGAM, but with no effects on overall maltase activity of the intestinal epithelium. Overall results show that there is a potential to change the rate of digestion of starches, starch products, and other saccharides like sucrose by phenolics present in the diet. Each tested polyphenol displayed a distinctive pattern of inhibition in the MGAM and SI subunits, as well as variations in the relative potency of the enzymes derived from human, rat or mouse species. These results show that dietary phenolic compounds cause differential or selective inhibition of the different intestinal α-glucosidase activities. Of relevance is the finding that some phenolic compounds modify patterns of protein forms expression. We speculate that different alternative spliced forms of MGAM are present to digest starch efficiently in the presence of polyphenolic inhibitors.

CHAPTER 1. INTRODUCTION

1.1   Background

For humans, diets which are rich in starches play a vital importance for human, because they provide energy for the body and in particular for the brain. Diets including significant amounts of starch are even more important for children as they provide an important source of glucose during the developmental stages, as well for high-energy performance (Williams, 1989). Even so, high carbohydrate diets have a negative reputation for the health because of an association with metabolic diseases such as Type 2 diabetes, obesity, cardiovascular (Ludwig, 2002). The occurrence of Type 2 diabetes has been increasing and it is estimated that 52% of the US population will have either diabetes or prediabetes by 2020 (de Bock et al., 2012). The Type 2 diabetes prevalence is estimated to reach 360 million by year 2030 in the world (Wild et al., 2004). Diets consisting of mainly low glycemic index (GI) carbohydrates are considered to be beneficial to Type 2 diabetics by slowing starch digestion and the accompanying glucose release profile in an extended time (Jenkins et al., 2008). Jenkins et al. (1981) first described the concept of GI which is the area of the postprandial blood glucose response profile in 2 h from a particular food as compared to a control, either glucose or white bread (Jenkins et al., 1981). It is categorized into two main groups: low GI foods (≤ 55) and high GI foods (≥ 70). Low GI foods have slower glucose release, whereas high GI

foods cause faster and greater glucose release into the bloodstream (Ludwig, 2002; Atkinson et al., 2008). In a similar way, Englyst and colleagues (Englyst et al., 1992; Englyst, 2005) devised a nutritional classification of starchy foods based on an in vitro analysis into rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS). Starch is the main glycemic carbohydrate (digestible) and can provide up to 70-80% of calories in the human diet (Whistler & BeMiller, 1997). RS cannot be digested; however, it is used by colonic bacteria which ferment them producing by products of short chain fatty acids (SCFA), hydrogen gas, methane, and carbon dioxide (Southgate, 1989).

During digestion in the human, α-amylase first degrades starch to linear glucose oligomers and branched α-limit dextrins that are further hydrolyzed into free glucose by the mucosal exoglucosidases Maltase-Glucoamylase (MGAM) and Sucrase-Isomaltase (SI) located on the small intestinal brush border membrane. The released glucose is then absorbed into blood circulation. Sucrose is digested by sucrase, but this enzyme has high glucogenic activity for starch α-amylase degradation products. The sodium-dependent glucose transporter 1 (SGLT1) transports the glucose into the epithelial cells and out into the portal vein for transport to the liver and elsewhere. Fructose is transported passively into the epithelial cells via GLUT5.

The control of glucose release from starch digestion is desired for moderating blood glucose excursions and includes the approach of inhibition of activities of intestinal mucosal α-glucosidases. Various compounds have been shown to have inhibitory effect at the α-glucosidase level and phenolics, in particular, have been of interest since they are natural compounds derived from plants and they differ in their inhibitory potencies.

1.2 Thesis Overview and Objectives

In this thesis work, it was hypothesized that selective or differential inhibition of the individual subunits of MGAM and SI by specific phenolic compounds can produce different digestion rates of starch, starch products, and sucrose for moderating the glycemic response profile. For example, higher inhibition of glucoamylase, the fastest acting enzyme, may slow digestion for glucose release into the distal small intestine where it may have further effect of triggering appetitive response through the entero-endocrine L-cells. Likewise, significant inhibition of sucrase coupled with a high sucrose-containing food would slow its digestion. The focus of each chapter of the thesis is listed as follows:

Chapter 1 introduces the background of carbohydrate, starch, and starch digestion.

Chapter 2 reviews the literature about starch digestion, starch digesting enzymes, MGAM genes and synthesis, SI genes and synthesis, catalytic characteristics of MGAM and SI, and starch digesting enzymes inhibitors.

Chapter 3 focuses on determination of the selective inhibition of the individual recombinant subunits (C, Ct and N, Nt terminal) of MGAM and SI using known polyphenolic α-glucosidase inhibitors; caffeic acid, gallic acid, chlorogenic acid, (+)-catechin, and (-)-epigallocatechin gallate (EGCG). These studies have revealed that inhibition of the highly active Ct-MGAM or called as glucoamylase by EGCG and chlorogenic acid, and with lesser inhibition of the slower acting enzymes, may create a desired slow starch digestion.

Chapter 4 describes the inhibitory potencies of same phenolic compounds used in the Chapter 3 for the inhibition of maltase, sucrase and isomaltase activities of rat intestinal acetone powder. In this study, inhibition mechanisms, inhibition constants, as well IC_{50} values, were determined for maltase, sucrase, and isomaltase activities of rat intestinal acetone powder. These studies have revealed that EGCG and chlorogenic acid demonstrate the highest inhibitory capacities for maltase, sucrase and isomaltase.

Chapter 5 explores the effects of phenolic compounds (caffeic acid, gallic acid, (+)-catechin) on expression levels of MGAM mRNA and SI mRNA in mouse jejunal small intestine explants, their protein expression, and maltase activities of homogenized mouse jejunal small intestinal explants. This study has showed that some of the tested phenolic compounds modulate the expression of MGAM and SI mRNA, and also resulted in the production of different molecular size forms of MGAM, but these changes had no effect on overall maltase activity of the intestinal epithelium.

Chapter 6 examines the inhibitory potencies of selected phenolic compounds used in Chapter 1 on maltase activity of human small intestinal lysate, and immunoprecipitated MGAM (MGAM-IP) and immunoprecipitated SI (SI-IP), as well as sucrase activity of (SI-IP). This study revealed, with difference in intensity compared to the above work, that sucrase activity of SI-IP was highly inhibited by chlorogenic acid, whereas EGCG inhibited the maltase activity of MGAM-IP. Therefore, these compounds may be effective compounds to change the glucose release in the human small intestine.

Chapter 7 reports an overall thesis summary with the major findings, future work opportunities, and comparisons of findings.

CHAPTER 2. REVIEW OF LITERATURE

2.1 <u>Starch digestion and starch digesting enzymes</u>

Starchy foods are major components of the human diet, and often comprise about 50% of the energy intake. These foods are derived from cereals and tubers, and overall come mainly from corn, rice, wheat, and potatoes. Starches are lastly digested into glucose by the activity of the α-glucosidases present in the gastrointestinal tract. The glucose released by this process is absorbed by the intestinal epithelial cells and then deposited into the blood stream to be subsequently used as a major energy source for the human body. Starch is composed of glucose oligomers with two main structural forms: amylose and amylopectin. While amylose consists mostly of a linear α-1,4-linked glucose polymer (500-600 glucose residues); amylopectin has a short linear α-1,4-linked glucose polymer segments (12-70 glucose residues) with a relatively large proportion of α-1,6-links that produces a branched structure. The starch digesting enzymes (α-amylase, MGAM and SI) belong to glycoside hydrolases (GH) enzyme family which hydrolyzes the glycosidic bond between two carbohydrate molecules. A large number of various GH families based on the amino acid sequence similarity (Henrissat, 1991) are collected in Carbohydrate Active Enzyme database (CAZY).

Starch is digested first by salivary and pancreatic α-amylases, also known as α-1,4-endoglucosidases (EC 3.2.1.1), cleaving the internal α-1,4 bonds of amylose and amylopectin with production of the linear oligomers of maltose, maltotriose, and maltotetraose, and the branched α-limit dextrins (LDx). Only a very small proportion of free glucose is produced (Quezada-Calvillo et al., 2008). The salivary and pancreatic α-amylases are of the well-known family GH13 hydrolases. The linear oligomers and LDx are subsequently and extensively degraded to free glucose by the exoglucosidases MGAM (EC 3.2.1.20 and 3.2.1.3) and SI (EC 3.2.148 and 3.2.10). These enzymes belong to GH31 family and act on the non-reducing end of their substrates from which glucose is released. The GH31 family is composed of four subgroups formed by their characteristic amino acid sequences (Ernst et al., 2006). All subunits of the mucosal α-glucosidases (MGAM and SI) are in the subgroup 1 which shares the WIDMNE sequence in the catalytic center of the protein structure.

MGAM and SI display differences in terms of their glucosidic activities. MGAM has α-1,4 and small amount of α-1,6 glucosidic activity. In contrast, the sucrase subunit of SI has α-1,2 and α-1,4 glucosidic activity, while the isomaltase subunit of SI possesses α-1,4 and α-1,6 glucosidic activity cleaving the linkages in the branching points of intermediate branched oligomers. Each of these enzymes is composed of two subunits (C, Ct and N, Nt terminals) with the catalytic sites acting on the non-reducing ends of linear segments of glucose oligomers with the release of free glucose. Finally, the released glucose is absorbed into blood circulation. SGLT1 transports the glucose or galactose into the epithelial cells by crossing the apical membrane of epithelial cells.

Fructose is transported via GLUT5 into the epithelial cells by a passive diffusion mechanism.

2.2   Maltase-Glucoamylase gene and protein synthesis

Human MGAM is 1857 amino acid long with the molecular size of approximately 210 kDa (Nichols et al., 1998) and is encoded by a gene located on the chromosome 7 (Nichols et al., 2003). Different species which have been sequenced show similar gene arrangements and a different number of tandem segments encoding for the C terminal subunit (Naumoff, 2007). In humans, the MGAM gene contains two tandem segments each coding for a whole C terminal subunit (UCSC Genome Browser). In mice, the MGAM gene is located on chromosome 6 and consists of four different glucohydrolytic subunits. In mice, the N terminal of the mature protein is the first region at the 5' extreme and is anchored to the membrane. Three tandem regions each coding for a C-terminal subunit are followed by the N-terminal. The N and C terminal are then followed by a segment representing the stop codons giving stop signals for the translation of the protein. Since the MGAM gene can contain multi-C terminal subunits, this can result in RNA alternatively spliced to generate different forms of MGAM. There have been shown two spliceforms from mice (CtMGAM-N2 and CtMGAM-N20) that were recombinantly expressed as seen in Figure 2.1 (Jones et al., 2011). To make mature MGAM, the encoded protein experiences extensive *N-* and *O-*glycosylation.

MGAM protein is composed of five protein domains: a small cytosolic domain as seen in Figure 2.2, a transmembrane domain (TMD), a O-glycosylated linker (O-link),

and two homologous catalytic subunits (C and N terminal) (Jones et al., 2011). In the cytosolic domain, MGAM has 26 amino acids with 5 lysine residues. It contains a hydrophobic segment with 16 branched amino acids (Nichols et al., 1998). In the transmembrane domain, MGAM has approximately 12 amino acids long of a hydrophobic segment (Nichols et al., 1998). The O-glycosylated linker is 52 amino acids long with 20 potential O-glycosylation sites and rich in threonine and serine (Nichols et al., 1998). O-glycosylate linker links the N terminal of MGAM to the brush border membrane. C and N terminal of MGAM is approximately 900 amino acids long (Nichols et al., 1998). C terminal subunits of MGAM locate at the luminal side (Engelman & Steitz, 1981) while N terminal is anchored to the brush border membrane (Hauri et al., 1982).

MGAM in the rat small intestine was shown to have two subunits of molecular weight of 130 and 145 kDa (Lee et al., 1980; Lee & Forster, 1984). MGAM is synthesized as a single chain polypeptide precursor with a molecular weight of 240 kDa (Noren et al., 1986). It is cleaved into two separate subunits (C terminal, glucoamylase subunit and N terminal, maltase subunit) by pancreatic proteases at the apical membrane surface (Noren et al., 1986). However, Hauri et al. (1985) used highly specific monoclonal antibody and found that human MGAM has an apparent molecular weight of 355 kDa. They also proposed that MGAM does not undergo proteolytic processing in the microvillus membrane and this high molecular weight of MGAM might occur because of the extensive possible glycosylation. Another study reported that there is no evidence showing that human MGAM undergo proteolytic processing and this is due to the high O- and N-linked glycosylation hampering enzymatic proteolysis (Naim et al., 1988).

The amount of MGAM molecules is less than the SI molecules in the apical membrane of epithelial cells and it covers approximately 2% of brush border membrane proteins (Van Beers et al., 1995). MGAM molecules are distributed along the small intestine and its activity is higher in the ileum (Asp et al., 1985; Dahlqvist, 1962). MGAM and SI have 59% similarity in their amino acid sequence (Figure 2.2). Both of C terminals and N terminals of MGAM and SI show 60% amino acid sequence similarity. The C and N terminal of MGAM or SI has 40% amino acid sequence similarity (Jones et al., 2011).

2.3   Sucrase-Isomaltase gene and protein synthesis

SI is 1827 amino acid long with the molecular size of approximately 210 kDa (Sigrist et al., 1975) and is encoded by a gene located on chromosome 3 (Chantret et al., 1992). The precursor form of the SI complex, Pro-SI, of a single polypeptide chain of 260 kDa, is used to produce the SI complex in the Golgi membrane (Hauri et al., 1979). Pro-SI is exposed to intracellular glycosylation (N- and O- glycosylation) (Danielsen et al., 1983) to render mature SI. After that, it is transported to the apical membrane of enterocytes where it is exposed to the extracellular pancreatic proteases to generate two separate subunits of free luminal C terminal (sucrase) and bound N terminal (isomaltase) to the brush border membrane. Although they are covalently separated, they interact with each other through noncovalent interactions (Semenza, 1981). The intracellular processing of SI protein is critical in the transportation of SI molecules to the apical surface of the brush border membrane, since once SI protein is correctly processed intracellularly, it can be sensed by cytosolic transporters. If not, as has been shown in the

case of mutations of SI protein, its transport of SI molecules to the apical side of enterocytes can be impaired resulting in starch digestion problems; as seen in SI genetic deficiencies in humans (Ouwendijk et al., 1998; Ritz et al., 2003).

SI protein consists of five protein domains: a small cytosolic domain, a transmembrane domain (TMD), an O-glycosylated linker (O-link), and two homologous catalytic subunits (C and N terminal as shown in Figure 2 (Jones et al., 2011). The cytosolic domain of SI is 12 amino acids long involving a negative and three positively charged amino acids. In the transmembrane region, SI is 13-32 amino acids long. The O-glycosylated domain of SI is 33-70 amino acids long which binds the N-terminal subunit to the transmembrane. This domain has a high number of threonine and serine residues. These amino acids attach to fucose, galactose, and hexosoamine by O-glycosylation to block proteolytic hydrolysis in vivo (Kelly & Alpers, 1973). C- and N-terminals of SI together have approximately 900 amino acids. As similar to MGAM, C terminal and N terminals of SI are located at the luminal side and bound to the brush border membrane, respectively.

SI represents 10% of brush border membrane proteins (Van Beers et al., 1995) and is distributed along the length of the small intestine. Its highest activity is in the jejunum part (Asp et al., 1985).

In humans, SI is the predominant α-glucosidase in the apical membrane of epithelial cells; MGAM compensates for its relative low abundance with a higher hydrolytic activity (Robayo-Torres et al., 2006).

2.4   Catalytic characteristics of Maltase-Glucoamylase

Dahlqvist (1962) first described the different catalytic properties of MGAM and SI and named them as Maltase II and Maltase III for glucoamylase (C terminal of MGAM) and maltase (N terminal of MGAM), and Maltase Ib and Maltase Ia for sucrase (C terminal of SI) and (N terminal of SI), respectively. Another study clearly showed that Maltase II corresponds to glucoamylase activities using monkey intestinal MGAM (Seetharam et al., 1970). Several studies used the difference in the heat stability of the C and N terminals of MGAM to allow for activity of N terminal subunit while suppressing the activity of the C terminal subunit due to its denaturation (Heymann & Gunther, 1994; Gunther et al., 1996), and to define the activities of individual subunits. These studies proposed that N and C terminal subunits had the same activity. Another study reported that MGAM had both α-1,4 and α-1,6 linkage hydrolysis activity; however, it hydrolyzed α-1,4 linkages faster than α,1-6 linkages (Kelly & Alpers, 1973). It has been shown that the MGAM complex had a higher binding affinity, lower Michealis Menten constant (Km), for longer oligosaccharides (Heymann & Gunther 1994; Heymann et al., 1995). Also, Quezada-Calvillo et al. (2008) showed that Ct-MGAM has high affinity for longer maltooligasaccharides. Two properties explaining the high affinity of Ct-MGAM for longer maltooligosaccharides were shown to be Phe instead of Ala and the 21 amino acid extension. Contrary to Ct-MGAM, Nt-MGAM had a higher affinity for hydrolyzing shorter oligosaccharides (Quezada-Calvillo et al., 2008). The presence of maltotriose and maltotetraose caused a strong substrate inhibition of α-glucogenic activity of immunoprecipitated MGAM (Figure 2.3) (Quezada-Calvillo et al., 2008). This finding

demonstrated that MGAM can be fully active under low concentrations of maltotriose and maltotetrose. In addition, both Ct-MGAM (recombinantly expressed CtMGAM-N2 and CtMGAM-N20) clones experienced strong substrate inhibition with maltotriose (Jones et al., 2011). This finding was supportive to previous studies showing the substrate inhibition by maltotriose and weak inhibition by maltotetraose on the activity of MGAM complex (Heymann & Gunther 1994; Heymann et al., 1995). Immunoprecipitated MGAM demonstrated similar kinetics to Ct-MGAM by a similar affinity or Km values for maltose and substrate inhibition caused by maltotriose (Quezada-Calvillo et al., 2008). Therefore, these results indicated that Ct-MGAM represents the majority of IP-MGAM α-glucosidic activity. Some researchers showed that mouse Ct-MGAM had relatively higher activity for digesting cooked starch at even low enzyme units (5 units) among all subunits (Lin et al., 2012). Figure 2.4 demonstrates that the highest starch digesting capacity was obtained with Ct-MGAM compared to other subunits. Nt-MGAM demonstrated weak substrate inhibition with maltose, maltotriose and maltotetraose. Nt-MGAM did not show any substrate inhibition when it was tested either with maltodextrins or limit dextrins. Ct-MGAM has been reported the most active enzyme and even digests whole undigested starch molecules as shown in Figure 4 (Lin et al., 2012).

2.5   Catalytic characteristics of Sucrase-Isomaltase

Semenza (1981) showed that SI has all of the sucrase activity, 80% of the total intestinal maltase activity, and nearly all the isomaltase activity (Semenza, 1981). Another more recent study demonstrated that immunoprecipitated SI accounted for 72%

of maltase activity, 99% of sucrase activity, and 95% of palatinase activity due to the α-1,6 activity of the isomaltase subunit (Quezada-Calvillo et al., 2007). Although both MGAM and SI have maltase activity, immunoprecipitated MGAM has a much higher activity (lower Km value of 2.7 ± 0.3 mmol/L) than immunoprecipitated SI (Km value of 35.8 ± 2.0 mmol/L). Unlike MGAM, SI did not experience any substrate inhibition in the presence of maltose, maltotriose, maltotetraose, and maltopentaose (Quezada-Calvillo et al., 2008).

Although Ct-SI and Nt-SI demonstrate maltase activity, Ct-SI has additional sucrase activity and Nt-SI has additional isomaltase activity (Hauser & Semenza, 1983; Heymann et al., 1995). While Ct-SI has more α-1,4 activity, Nt-SI has a significant α-1,6 debranching activity when limit dextrin was used as a substrate (Gray et al., 1979). Another study supported this by showing that the Ct-SI subunit hydrolyzes sucrose and maltose, while Nt-SI had the essential isomaltase activity (Heymann et al., 1995).

2.6   Starch digesting enzyme inhibitors

A number of studies have recently focused on the controlling the free glucose release from starch by inhibiting salivary and pancreatic α-amylases or the intestinal α-glucosidases (Jones et al., 2011; Nichols et al., 2009). For non-insulin-dependent Type II diabetics, the inhibition of these enzymes, in particular the intestinal α-glucosidases, reduces postprandial hyperglycemia and hyperinsulinemia, decreases the occurrence of insulin resistance, and also the stress on β-cells (Jacob, 1995; Chiasson et al., 2002; Scheen, 2003; Simpson et al., 2003).

The first marketed α-glucosidase inhibitor was acarbose in the early 1990s (Krentz & Bailey, 2005). Later, voglibose in Japan in 1994 and miglitol in the United States in 1996 were introduced into the market (Krentz & Bailey, 2005). Acarbose, miglitol, voglibose, and 1-deoxynojirimycin (DNJ) are available α-glucosidase inhibitors (Figure 2.5). Jones et al. (2011) tested the inhibitory effects of two pharmaceutical α-glucosidase inhibitors, acarbose and miglitol, on the individual subunits (C and N terminals) of MGAM and SI. While acarbose exhibited stronger inhibition on recombinant Ct-MGAM-N2 and Ct-MGAM-N20 (different alternative splices forms) and Ct-SI with Ki values of 0.009, 0.028 μM, and 0.246 μM, respectively; its inhibitory potency was much lower for Nt-MGAM and Nt-SI with Ki values of 14 μM and 62 μM (Jones et al., 2011; Sim et al., 2010). Acarbose also selectively inhibited the individual subunits of α-glucosidases when α-limit dextrin was used as the substrate (Lee et al., 2012). For instance, Ct-MGAM and Ct-SI were inhibited efficiently by acarbose at 5 nM while Nt-MGAM and Nt-Si were not inhibited at the same concentration (Lee et al., 2012).

Miglitol inhibited Ct-MGAM-N2 and Ct-MGAM-N20, and Ct-SI with Ki values of 0.211 μM and 0.230 μM and 0.130 μM, whereas its inhibition constant for Nt-MGAM and Nt-SI were 1 and 0.148 μM. Another study showed the inhibition constant (Ki) of voglibose for Ct-MGAM and Nt-MGAM as 3.31 μM and 2.22 μM, respectively (Ren et al., 2011). They also found that Ki values of DNJ were 2.04 μM and 1.41 μM for Ct-MGAM and Nt-MGAM, respectively (Ren et al., 2011). Acarbose, miglitol, voglibose and DNJ inhibited Ct-MGAM and Nt-MGAM in a competitive way (Figure 8). Earlier studies showed that acarbose inhibited 90% of glucoamylase, 65% of sucrase, 60%

of maltase, and 10% of isomaltase (Bishoff, 1994; Mooradian & Thurman, 1999). However, these strong inhibitors resulted in high amounts of undigested glycemic carbohydrates to enter the colon resulting in abdominal discomfort and/or diarrhea (Krentz at al., 1994; Krentz & Bailey, 2005).

Other compounds such as salacinol, kotalanol, blintol and de-*O*-sulfonated kotalanol of *Salacia reticulata*, plant found in Sri Lanka and South India are used for treating Type 2 diabetes (Muraoka et al., 2008; Jones et al., 2011). Their structures are slightly different and these changes rendered them to selectively inhibit the individual subunits of the intestinal α-glucosidases (Jones et al., 2011). Thus, these compounds showed their strongest inhibitory effects on different subunits of MGAM and SI, such that salacinol, kotalanol, blintol and de-*O*-sulfonated kotalanol inhibited maltase activity of Ct-SI, Ct-MGAM, Ct-SI and Nt-SI with Ki values of 0.047 μM, 0.013 μM, 0.042 μM and 0.012 μM, respectively.

Naturally-found phenolics are one group of chemical metabolites reported to have inhibitory effect on the α-glucosidases. Chlorogenic acid was recognized as the major α-glucosidase inhibitor in the leaf extract of *Nerium indicum,* and it indicated a non-competitive inhibition mechanism for maltase and sucrase activities with IC_{50} values of 2.99 and 2.18 mM, respectively (Ishikawa et al., 2007). Also, it was shown that caffeic acid was as potent as a rat intestinal α-glucosidase inhibitor among different cinnamic acid derivatives (Adisakwattana et al., 2009); however, its inhibitory potency was low for maltase and sucrase with IC_{50} values of 0.74 and 0.49 mM, respectively (Welsh et al., 1989). EGCG, the main catechin found in green tea (Forester et al., 2012), was shown as a potent inhibitor of maltase activity in rat brush border membrane vesicles (Kamiyama

et al., 2010), using rat intestinal acetone powder (Matsui et al., 2007), and as well as using recombinant human intestinal maltase (Nguyen et al., 2012). EGCG inhibited α-glucosidase of rat intestinal acetone powder with an IC_{50} of 914.8 µmol/L (Xu et al., 2013). In another study, EGCG was found to be competitive inhibitor and its IC_{50} was estimated as 20 µM for human intestinal maltase (Nguyen et al., 2012). The oxidized form of gallic acid inhibited nearly 40% of rat brush border sucrase activity (Welsh et al., 1989); and, in another study, gallic acid was a potent inhibitor for sucrase and maltase (Gupta et al., 2007). Fruits rich in anthocyanin showed a strong inhibition for α-glucosidase activity (McDougall et al., 2005). Sucrase and maltase were inhibited by cyanidine-3-*O* rutinoside with IC_{50} values of 0.25 mM and 2.3 mM, respectively (Adisakwattana et al., 2011).

Changes in postprandial glucose with several phenolics have been studied in animal and human studies. Postprandial glucose response to maltose was reduced by theaflavin 3-*O*-gallate and 6-*O*-caffeoylsophorose (Matsui et al., 2004; Matsui et al., 2007). The plasma glucose level after maltose or glucose administration in mice was reduced with a crude Acerola polyphenols fraction consisting of anythocyanins (Hanamura et al., 2006). Cocoa proanthocyanidins resulted in the prevention of the increase of blood glucose in genetically diabetic obese mice (Tomaru et al., 2007). Blood glucose response was reduced with the extract of Cassia auriculata, a herb from Sri Lanka, and this was found when maltose was provided but not sucrose or glucose (Abesundara et al., 2004). Postprandial rise in blood glucose to maltose or sucrose administered rats was reduced with a leaf extract of *Nerium indicum* (Ishikawa et al., 2007). Blood glucose and insulin response to maltose was reduced in rats when

diacylated anyhocyanin and anthocyanin extract of purple sweet potato were given (Matsui et al., 2002).

The effect of polyphenols on glucose generation or absorption in the small intestine has been tested also in polyphenol-containing drinks. A change in postprandial glycemic response was obtained in twelve subjects when 35 g of sucrose was taken with 150 g of puree of billberries, blackcurrants, cranberries and strawberries (Johnston et al., 2002). Another study showed that a different postprandial glycemia pattern was obtained as a result of the consumption of cranberry juice consisting of high-fructose corn syrup as a sweetener when compared to the control without any cranberry juice (Wilson et al., 2008). Furthermore, gastrointestinal hormone (GIP and GLP-1) profiles showed that there was a delay in the intestinal absorption in nine healthy subjects after ingestion of glucose (25 g) with coffee (400 ml with 350 mg chlorogenic acid) (Johnston et al., 2003). They proposed that chlorogenic acid could change the rate of intestinal glucose absorption or changing the location of glucose absorption to more distal parts of the intestine. In another study, glycemic response reduced with the ingestion of 25 g of sucrose in the instant coffee enriched with chlorogenic acid (Thom, 2007). It has been also shown that tea catechins, especially EGCG presents the potential for treating Type 2 diabetes (Kao et al., 2006). Also, the anti-diabetic effects of green tea catechins, in particular EGCG, in human studies were reported (Thielecke & Boshmann, 2009). Another study observed the relationship between the consumption of green tea and the risk for the Type 2 diabetes. Subjects who consumed more than 6 cups of green tea daily were less at risk for Type 2 diabetes compared to subjects who drank less than a cup per week (Iso et al., 2006).

2.7   <u>References</u>

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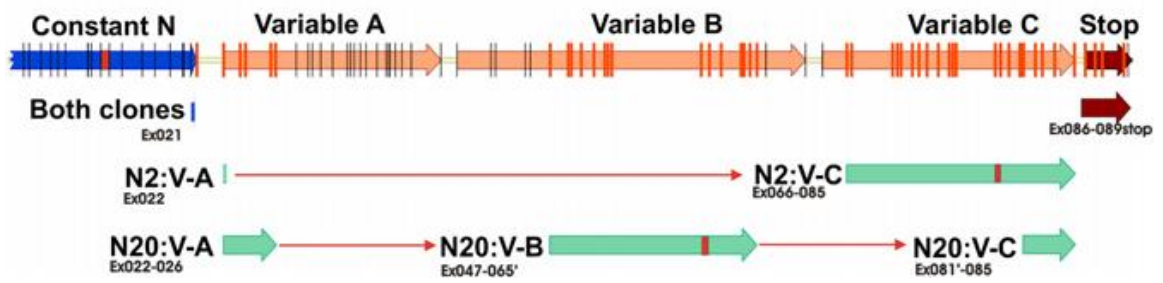
Figure 2.1 Clones of mouse Ct-MGAM with alternative splicing. Genomic map diagram is presented at the top. Map of transcribed segments present in the individual clones of N2 and N20 is shown by green arrows. The number of involved exons is given in black below the the clone segments. The location of the GH31 signature sequence WIDMNE is presented with red vertical bars (Figure source: Jones et al. 2011).
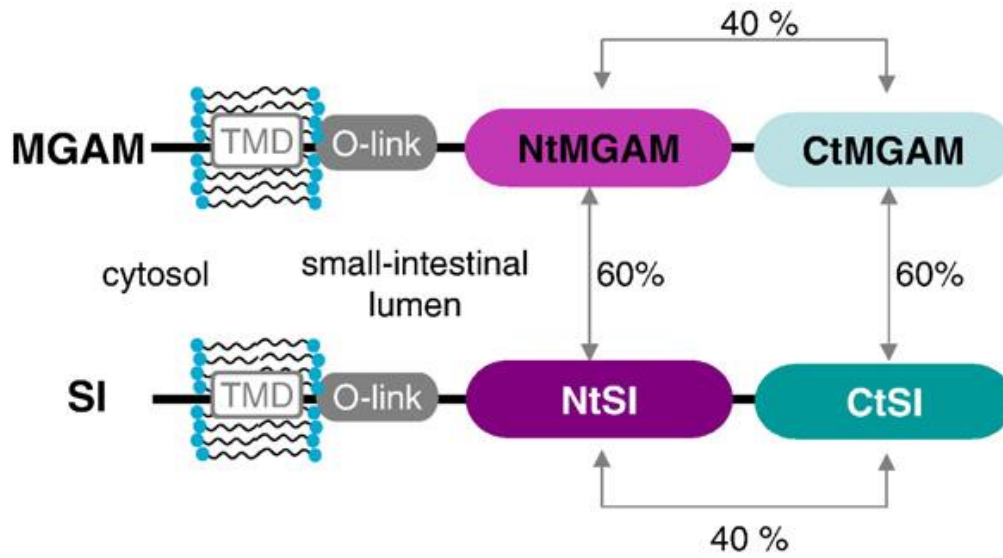
Figure 2.2 Protein organization of MGAM and SI is represented linearly. The similarity in the amino acid sequence of catalytic subunits is given as percentage (Figure source: Sim et al. 2008).
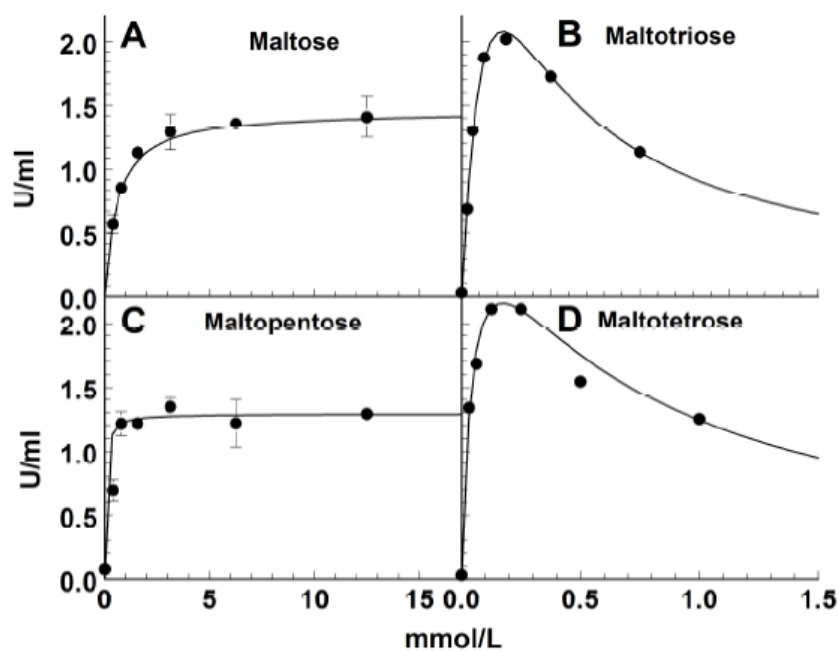
Figure 2.3 Kinetic pattern of the α-glucosidic activity of immunoprecipited MGAM against maltose (A), maltotriose (B), maltopentose (C) and maltotetrose (D) (Figure source: Quezada-Calvillo et al. 2008).
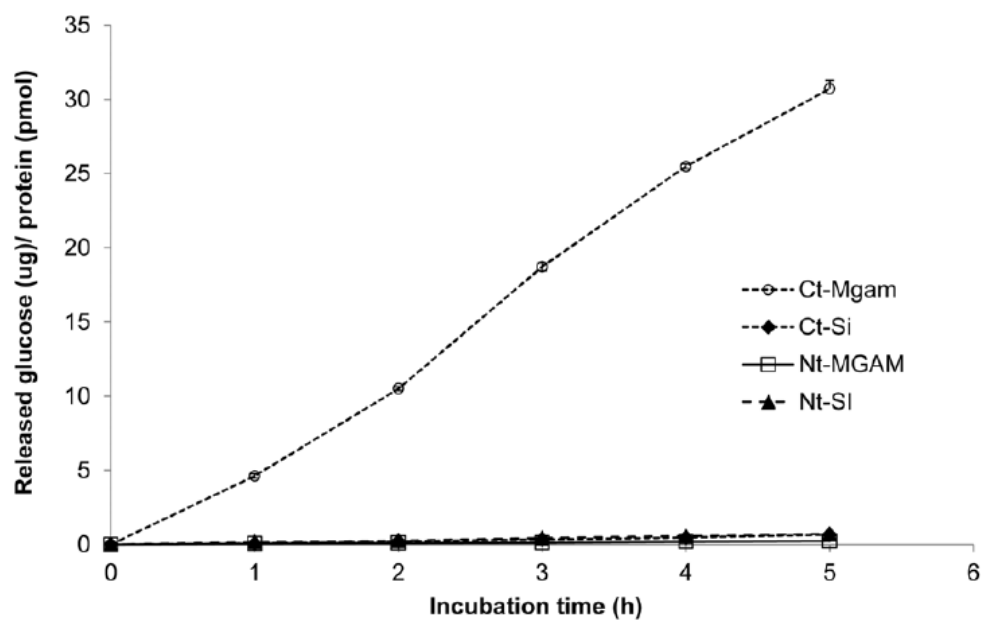
Figure 2.4 Direct starch digestive capability of individual mucosal α-glucosidases. Enzyme amount, 30 units (Figure source: Lin et al. 2012).
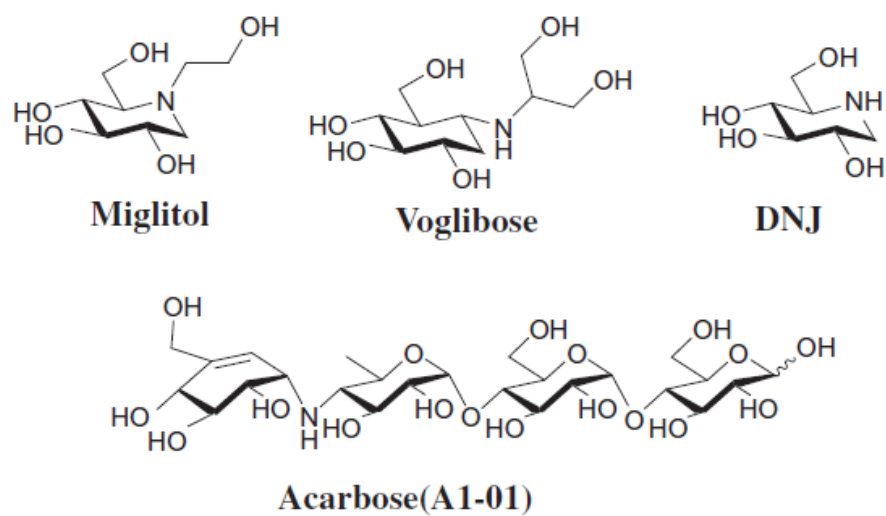
Figure 2.5 Structures of α-glucosidase inhibitors: acarbose, miglitol, voglibose and DNJ (Figure source: Ren et al. 2011.

CHAPTER 3. DIETARY PHENOLIC COMPOUNDS SELECTIVELY INHIBIT
ACTIVITIES OF INDIVIDUAL SUBUNITS OF MALTASE-GLUCOAMYLASE AND
SUCRASE-ISOMALTASE FOR MODULATING GLUCOSE RELEASE

3.1   Abstract

Some phenolic compounds have been shown to inhibit the activity of the
intestinal α- glucosidases, Maltase-Glucoamylase (MGAM) and Sucrase-Isomaltase (SI).
Their effect on the individual C and N terminal (Ct, Nt) subunits of MGAM and SI could
help modulate glucose release from starch digestion in the small intestine by selectively
inhibiting the most active subunits, i.e. Ct-MGAM and Ct-SI, while still allowing the
slower digesting subunits to digest starch. The phenolic compounds caffeic acid, gallic
acid, (+)-catechin, chlorogenic acid, and (-)-epigallocatechin gallate (EGCG) were used
with recombinant human Nt-MGAM and Nt-SI, and mouse Ct-MGAM and Ct-SI, to
determine the inhibition of their enzymatic activity using maltose. Glucose release was
measured by the Tris glucose-oxidase peroxidase method. IC_{50} values, the inhibition
mechanisms, and respective inhibition constants for the each subunit were determined.
EGCG and chlorogenic acid were found to be the most potent inhibitors, particularly for
Ct-MGAM and Ct-SI. All compounds displayed non-competitive type inhibition.
Selective inhibition of Ct and Nt α-glucosidase subunits was obtained by the use of
specific phenolic compounds. Inhibition of the highly active Ct-MGAM by EGCG and

chlorogenic acid could create a slow starch digestion effect for improved glycemic response with potential health benefit.

Key words: α-glucosidases, inhibition, Maltase-Glucoamylase, phenolics, Sucrase-Isomaltase.

3.2   Introduction

Starchy foods are a major component of the human diet for energy production. Starch is digested into the glucose by six different enzymes: salivary and pancreatic α-amylase and the α-glucosidases, Maltase-Glucoamylase (MGAM) and Sucrase-Isomaltase (SI), each composed of two active subunits located on the respective C and N terminals (Ct, Nt) of the original protein. The first step in starch digestion is performed by the α-1,4-endoglucosidases, salivary and pancreatic α-amylases, with production of linear glucose oligomers and branched α-limit dextrins, and very little free glucose (Quezada-Calvillo et al., 2008). The α-amylase degradation products are further hydrolyzed into free glucose by the mucosal α-1,4 exoglucosidases MGAM and SI located on the small intestinal brush border membrane (Quezada-Calvillo et al., 2007). MGAM and SI enzyme complexes belong to the GH31 family of glucohydrolases. The Nt of both MGAM and SI attach these enzyme complexes to the apical membrane of small intestinal enterocytes through an O-glycosylated stalk domain (Sim et al., 2008). Each subunit of MGAM and SI has different α-glucosidic catalytic properties related to their independent active sites; therefore, their contribution to glucogenesis of glycemic carbohydrates is different (Quezada-Calvillo et al., 2008; Lin et al., 2012).   All four subunits have high α-1,4-exoglucosidic activity (Jones et al., 2011; Auricchio et al.,

1965). In addition, Ct-SI and Nt-SI subunits display distinctive sucrase and isomaltase activities. Ct-MGAM and Nt-MGAM share activities against linear glucose oligomers, but the higher activity of Ct-MGAM on longer glucose oligomers led to the naming of this subunit as glucoamylase, while Nt-MGAM has been ascribed as the maltase subunit (Quezada-Calvillo et al., 2008). Ct-MGAM recently has been shown the most active enzyme and even digests whole, undigested starch molecules (Lin et al., 2012). Others have shown its high maltase activity compared to the other subunits (Jones et al., 2011; Auricchio et al., 1965). In humans, although SI is the predominant molecule in the apical membrane of epithelial cells, MGAM compensates its relative low abundance with a higher hydrolytic activity (Robayo-Torres et al., 2006).

A strategy to attain control of the rate of free glucose release from starch is to selectively modulate inhibition of the hydrolytic activity of salivary and pancreatic α-amylases, or of the intestinal α-glucosidases where glucose is actually generated. We recently showed that acarbose selectively inhibited Ct-MGAM and SI for realizing a slower digestion of the α-amylase degradation products of starch (Lee et al., 2012). Slow digestion of starch by partial inhibition of intestinal α-glucosidases may have multiple positive health implications related to control of the glycemic response profile and perhaps eliciting the ileal brake and gut-brain axis response to reduce appetite and food intake (Lee et al., 2013). The inhibition of α-glucosidic activity of specific subunits of MGAM and SI can be considered as a key approach for controlling glucose release, because their catalytic activities are responsible for producing virtually all the free glucose available for intestinal absorption.

A variety of chemical compounds have been shown to have inhibitory effect on the α-glucosidases. These compounds are derived either from chemical synthesis (Eskandari et al., 2011; Eskandari et al., 2011), or from natural sources such as fruits (McDougall et al., 2005; Kim et al., 2005) or plants (Oku et al., 2006; Ryu et al., 2010; Ramkumar et al., 2010; Li et al., 2010). For instance, caffeic and chlorogenic acids are typical hydroxycinnamic acids found in high concentration in coffee beans (Clifford et al., 1987; Clifford et al., 2006). Naturally-found phenolic compounds are one group of chemical metabolites reported to have inhibitory effect on the α-glucosidases. Chlorogenic acid was identified as the major α-glucosidase inhibitor found in the leaf extract of *Nerium indicum* (Ishikawa et al., 2007). Caffeic acid was shown as a potent rat intestinal α-glucosidase inhibitor among different cinnamic acid derivatives (Adisakwattana et al., 2009). The oxidized form of gallic acid inhibited nearly 40% of rat brush border sucrase activity (Welsh et al., 1989); and in another study, gallic acid was shown to be a potent inhibitor for sucrase and maltase (Gupta et al., 2007). EGCG, the main catechin found in green tea (Forester et al., 2012), was observed to be a potent inhibitor of maltase activity in rat brush border membrane vesicles (Kamiyama et al., 2010), using rat intestinal acetone powder (Matsui et al., 2007), as well as using recombinant human intestinal maltase (Nguyen et al., 2012).

Overall, this information suggests that phenolic compounds are good candidates for the study of selective and differential inhibition of the individual subunits of MGAM and SI to modulate digestion of starchy foods in a slow, but complete way. Although the inhibition of α-glucosidases by phenolics is well documented, the mechanism of inhibition and their relative potency on the individual α-glucosidases have not been

reported. In the present study, we analyzed the potential for the selective inhibition of the individual Ct and Nt subunits of MGAM and SI, using known polyphenolic α-glucosidase inhibitors; caffeic acid, gallic acid, chlorogenic acid, (+)-catechin and (-)-epigallocatechin gallate (EGCG). The corresponding mechanism of inhibition and inhibition constants were also determined.

3.3   Methods and Materials

3.3.1   Materials

Caffeic acid, gallic acid, chlorogenic acid, (+)-catechin, and (-)-epigallocatechin gallate were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were obtained from Sigma-Aldrich.

3.3.2   Enzyme preparation

Cloning, expression, and purification of recombinant mouse Ct-MGAM (Glucoamylase subunit; spliceform N20), recombinant mouse Ct-SI (Sucrase subunit) (Jones et al., 2011), recombinant human Nt-MGAM (Maltase subunit) (Rossi et al., 2006), and recombinant Nt-SI (Isomaltase subunit) (Sim et al., 2010) were reported previously. Mouse Ct-MGAM and Ct-SI were generated by recombinant expression in baculovirus-Sf9 insect cell systems (Jones et al., 2011). Human Nt-MGAM and Nt-SI were expressed in *Drosophila S2 cells* (Rossi et al., 2006; Sim et al., 2010). Nickel-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).

3.3.3   Determination of protein concentration

Protein concentration in solutions was determined by the Bio-Rad Protein Assay kit (Hercules, CA, USA), using 10 µl of the protein solutions and 200 µl of Bradford dye reagent for at least 5 min. Absorbance was read at 595 nm in a Synergy HT microplate reader (BioTek; Winooski, VT, USA). Standards of bovine serum albumin were used in the concentration range of 31.25 to 500 µg/ml.

3.3.4   Enzyme and inhibition assays

Glucose release was measured by the Tris glucose oxidase (TGO) method (Quezada-Calvillo et al., 1993) modified for microplate wells, using 10 µl of the maltose solution at final concentrations ranging from 2.1 to 16.7 mM, mixed with 10 µl of the enzyme solution containing 0.2 mU of activity for Ct-MGAM, Ct-SI, and Nt-MGAM; and 0.1 mU of activity for Nt-SI. One unit (U) of enzyme activity was defined as the activity that cleaves one µmol of maltose per min. The mixtures were incubated for 1 h at 37 °C and then 180 µl TGO was added and incubated for an additional 45 min. Absorbance was read at 450 nm in the microplate reader. In order to correct for the scavenger effect of phenolics on the oxidative intermediates generated during the glucose oxidase-peroxidase reaction which can lead to possible misleading readings of optical density (OD) (Wong & Huang, 2014), curves of glucose concentration vs OD in the presence of different concentrations of each individual phenolic compound were constructed and used to adjust the OD obtained during the assay of inhibition of the

phenolics on the α-glucosidase enzymes. At the selected concentrations, phenolics caused less than 20% inhibition of the glucose oxidase-peroxidase developing reaction.

For inhibition assays, 10 µl of the phenolic inhibitor solutions were added to the reaction mixtures to attain final concentrations ranging from 0 to 666.7 µM. The final concentration range for the gallic acid, caffeic acid and (+)-catechin was 0-666.7 µM for the subunits; while chlorogenic acid and EGCG were used at a final concentration of 0-83.3 µM for C terminals of both MGAM and SI, and 0-666.7 µM for N terminals of MGAM and SI. Analyses were conducted in triplicate.

3.3.5 Determination of inhibition constant and inhibition mechanism

The inhibition mechanism and the respective inhibition constants (Ki) of each inhibitor for each enzyme subunit were determined by the best fit of non-linear regression analysis with the Levenberg–Marquardt algorithm using the Sigma Plot program (Systat Software Inc.; San Jose, CA., USA) and the standard mathematic equations describing the inhibition mechanisms for competitive, uncompetitive, non-competitive homogeneous, and non-competitive heterogeneous inhibition. The mathematical fit resulting in the best parameters of quality for each enzyme-inhibitor combination, was chosen as the most probable inhibition mechanism in each case. The significance value (p value) for the statistical analysis was chosen as less than 0.05. For the calculations of kinetic parameters we choose nonlinear regression methods instead of linear transformations and linear regression, to avoid the additional error introduced by the latter procedures (Leatherbarrow, 1990; Cornish-Bowden, 2014).

Figure 3.1 shows the assumed reaction mechanisms for each of the different inhibition types. In the competitive inhibition, the inhibitor binds the active site of the enzyme with a specific Ki value. The inhibitor binds the enzyme-substrate complex, intermediate product with a specific Ki value in the uncompetitive inhibition. In non-competitive homogeneous inhibition, the inhibitor binds to both the enzyme itself and enzyme-substrate complex with the same Ki value. On the other hand, in non-competitive heterogeneous inhibition, the inhibitor binds both the enzyme active site and enzyme-substrate complex with different Ki values (Ki_1 and Ki_2).The corresponding equations described below for fitting of the experimental data represent well-known inhibition mechanisms, where v is the rate of reaction, Vmax represents the calculated maximal rate of reaction, [S] is the substrate concentration, Km represents the calculated Michaelis constant, [I] is the concentration of the inhibitor (polyphenol), and Ki corresponds to inhibition constant.

Eqn. 1. Competitive Inhibition:

$$v = \frac{Vmax * [S]}{Km * \left(1 + \frac{[I]}{Ki}\right) + [S]}$$

Eqn. 2. Uncompetitive Inhibition:

$$v = \frac{Vmax * [S]}{Km + [S] * \left(1 + \frac{[I]}{Ki}\right)}$$

Eqn. 3. Non-competitive homogeneous inhibition:

$$v = \frac{Vmax * [S]}{(Km + [S]) * (1 + \frac{[I]}{Ki})}$$

Eqn. 4. Non-competitive heterogeneous inhibition:

$$v = \frac{Vmax * [S]}{Km * \left(1 + \frac{[I]}{Ki1}\right) + [S] * \left(1 + \frac{[I]}{Ki2}\right)}$$

3.3.6 Determination of IC_{50} values

The concentration of each polyphenolic compound needed to cause 50% inhibition of the activity of the individual enzyme subunits was defined as the IC_{50} value and calculated by lineal regression of the log transformed values of inhibitor concentration (log[I]) versus the relative activity (v/Vmax) at 16.67 mM maltose concentration, using Minitab 14 (State College, Pennsylvania, USA).

3.4 Results

3.4.1 Kinetics and mechanism of inhibition of the recombinant Ct-MGAM, Nt-MGAM, Ct-SI and Nt-SI

The inhibition capacity of the polyphenolic compounds, as well as the respective inhibition mechanism and inhibition constants were determined for each recombinant α-glucosidase subunit. The definition of the inhibition types is explained in methods and in Figure 3.1.

Caffeic acid showed non-competitive heterogeneous inhibition for Ct-MGAM, Nt-MGAM and Ct-SI, while it inhibited Nt-SI in a non-competitive homogeneous manner (Table 3.1). Caffeic acid demonstrated the higher binding affinity (lower

inhibition constant) for Ct-MGAM and the Ct-SI, as compared to N terminal enzyme subunits. The inhibition constants, Ki_1 and Ki_2 for Ct-MGAM were 56.5 and 191.8 μM while they were 58.2 and 323 μM for Ct-SI (Table 3.1).

Gallic acid exhibited the non-competitive heterogeneous mechanism for all four subunits (Table 3.1). As with caffeic acid, Ct-MGAM and Ct-SI were the most sensitive to inhibition by gallic acid, with values for Ct-MGAM of 24.9 and 50 μM for Ki_1 and Ki_2, and 31.3 and 142.8 μM for Ct-SI Ki_1 and Ki_2 , respectively (Table 3.1).

(+)-Catechin showed non-competitive heterogeneous inhibition on Ct-MGAM, Nt-MGAM, and Ct-SI, while it showed non-competitive homogeneous inhibition (Table 3.1) on Nt-SI. (+)-Catechin showed similar inhibition potency towards all four subunits (Figure 3.2). Affinities or inhibition constants of (+)-catechin for Ct-MGAM, Nt-MGAM and Ct-SI were in the range of 20.4 to 97.2 μM (Table 3.1). The value of of 33.7 μM (Table 3.1) for the Ki on Nt-SI indicated a higher inhibitory effect on this subunit compared to the other phenolics.

Chlorogenic acid showed non-competitive homogeneous inhibition for Ct-MGAM and Nt-MGAM, while Ct-SI and Nt-SI were inhibited in a non-competitive heterogeneous way (Table 3.1). Chlorogenic acid had higher inhibition binding affinities and correspondingly lower inhibition constants for Ct-MGAM (3 μM) and Ct-SI (1.8 and 28 μM), as compared to caffeic acid, gallic acid, (+)–catechin (Table 3.1). The binding affinity of chlorogenic acid was approximately 56 and 15 times greater affinity for Ct-SI and Ct-MGAM, than for Nt-SI and Nt-MGAM, respectively.

EGCG displayed a non-competitive heterogeneous inhibition mechanism for Ct-MGAM, Nt-MGAM, and Nt-SI, and non-competitive homogeneous inhibition for Ct-SI

(Table 3.1). Ct-MGAM was the most strongly inhibited subunit by EGCG, with Ki values of 1.7 and 6.5 µM (Table 3.1). EGCG, along with chlorogenic acid, were the most potent inhibitors for Ct-MGAM and Ct-SI. Figure 3.3 illustrates the change in the relative activities (v/Vmax) of Ct-MGAM, Nt-MGAM, Ct-SI, and Nt-SI with the different concentrations (0-666.7 µM) of EGCG in the presence of different maltose concentrations (2.1, 4.2, 8.3 and 16.7 mM). It was noticeable that Ct-subunits required two orders of magnitudes lower inhibitor concentration than the Nt-subunits. Thus, EGCG was a comparatively potent inhibitor for Ct-MGAM and Ct-SI (Figure 3.3 a and b). Although EGCG and chlorogenic acid differed in their inhibition types for Ct-MGAM, their Ki values were close to each other.

Overall these results show that EGCG and chlorogenic acid present a preference towards C terminals rather than N-terminals of MGAM and SI, but these two phenolics displayed notable differences in their inhibitory effect on the Ct-MGAM and Ct-SI. Highest inhibitory effect was observed on Ct-MGAM for EGCG, and on Ct-SI for chlorogenic acid. With exception of (+)-catechin which showed more inhibition of Nt-SI, there was a general trend toward higher binding affinities for Ct subunits over Nt. Since Ct-MGAM is the most active subunit towards starch among the four subunits (Lin et al., 2012), EGCG and chlorogenic acid are postulated to be effective in controlling glucose release in the small intestine. Chlorogenic acid, with its high inhibitory effect on Ct-SI, might have additional effect of moderating the rate of sucrose digestion.

3.4.2 IC_{50} values of inhibitors

In addition to the inhibition constants, IC_{50} values were also determined for the different polyphenolic compounds on the four α-glucosidase subunits. These values (Table 3.2) are not necessarily expected to be of same ranking as the inhibition constants, since IC_{50} presents the required concentration to inhibit 50% of the enzymatic activity. This value results from the overall inhibition effects of the different binding mechanisms that each compound may display. Caffeic acid showed IC_{50} values in the range of 137.4 to 266 μM (Table 3.2) for all of the α-glucosidase subunits; however, it did show a selectively stronger inhibition on Ct-MGAM and Nt-SI compared to the other two subunits (Figure 3.4). Gallic acid also showed stronger inhibition (lower IC_{50} values) on Ct-MGAM and Nt- SI than on the other subunits. (+)-Catechin showed 50% inhibition of Ct-SI and Nt-SI at a lower amount of 34.3 and 36 μM (Table 3.2 and Figure 3.4). Its inhibition for Nt-SI was much stronger than for other compounds, which is supported by its lowest inhibition constant for Nt-SI among the compounds. Chlorogenic acid and EGCG required the lowest concentration range (2.3-13.8 μM) to inhibit 50% of the activity of Ct-MGAM and Ct-SI among the compounds tested, while a higher concentration range (39.6-159.4 μM) was required for Nt-MGAM and Nt-SI (Table 3.2 and Figure 3.4). Therefore, chlorogenic acid and EGCG showed selective inhibition for C-terminals over N-terminals (Figure 3.4), in agreement with their high binding affinities (Ki).

3.5   Discussion and Conclusions

Phenolic acids and polyphenols have been known to inhibit the activities of the intestinal α-glucosidases, MGAM and SI (Clifford et al., 2006; Ishikawa et al., 2007; Adisakwattana et al., 2009; Welsh et al., 1989; Gupta et al., 2007; Forester et al., 2012; Kamiyama et al., 2010; Matsui et al., 2007). The use of phenolic compounds as modulators of the rate of glucose release from starchy foods has been proposed for the treatment of metabolic diseases such as diabetes (Hanhineva et al., 2010). Although there are some strong inhibitors of α-glucosidases, such as acarbose (Hanhineva et al., 2010), their adverse secondary effect is a major factor limiting their use. The purpose of this study was to explore the selective inhibition of individual MGAM and SI subunits by using phenolic and polyphenolic compounds to achieve a slow, but complete, glycemic carbohydrate (starch or sucrose) digestion in the small intestine. Stronger inhibitors, such as acarbose, typically cause high amounts of undigested glycemic carbohydrates to enter the colon resulting in abdominal discomfort and/or diarrhea. Additionally, slowly digestible starch likely triggers the ileal brake and gut-brain axis mechanism to control nutrient release rate from the stomach and affect hypothalamic regulated appetite control related to obesity and associated diseases (Lee et al., 2013; Maljaars et al., 2008; Strader & Woods, 2005).

Our findings show that the four mucosal α-glucosidases, SI and MGAM, are selectively and differentially inhibited using natural phenolics. This introduces the potential to induce different rates or profiles of digestion for starches, starch products, and sucrose. For instance, selective inhibition of the most active starch degrading subunit,

Ct-MGAM or Glucoamylase, was achieved with EGCG and chlorogenic acid. This implies that reducing activity of this enzyme could slow the rate of starch digestion and reduce the glycemic spike, as was proposed in a recent paper by our group (Lee et al., 2014). Chlorogenic acid also had the highest binding affinity (Ki_1) for Ct-SI or Sucrase. This suggests the possibility of slowing rate of digestion of sucrose rich foods. As another example, (+)-catechin showed higher inhibition for Nt-SI, the only subunit with endogenous α-1,6 branch hydrolyzing activity. Here, there is a possibility of combining (+)-catechin with highly branched starch or starch products, such as a highly branched maltodextrin (Lee et al., 2013), for a slow glucose release effect. Moreover, the results imply that the low inhibitory effect of phenolics on the slower acting Nt-MGAM and SI subunits may allow for starch to be digested in diets containing such phenolics, but avoiding the problem of stronger inhibitors affecting all four subunits that effectively reduce glycemic carbohydrate digestion and cause the problem of delivering large quantities of carbohydrates into the colon. .

The tested phenolics were shown to affect the ability of the substrate to bind the active site of all enzyme subunits (Ki_1); in some cases, phenolics may also bind already formed enzyme-substrate complex, although this with lesser affinity (Ki_2). It has been reported that the catalytic pockets of Ct-MGAM, Nt-MGAM and Nt-SI are surrounded by several hydrophobic aromatic amino acids, which may comprise the substrate binding motifs for these enzymes (Jones et al., 2011; Nguyen et al., 2012). Therefore, it may be speculated that phenolic compounds could interact and/or bind to these aromatic motifs in proximity to the active site, in turn impairing the binding of the substrate with a correct orientation. The substantially higher values for Ki_1 of phenolics than those found for

other competitive inhibitors (Lee et al., 2012) make unlikely the possibility for the existence of a typical competitive binding component in the interaction and therefore, discards the possibility of the existence of classic mixed type inhibition. Thus, the model of typical non-competitive inhibition was the one that most closely described the effects of phenolics. Therefore, phenolic and polyphenolic inhibitors bind to at least a second additional site. Mathematical fittings of the experimental data indicated that only five (Nt-SI/caffeic acid; Nt-SI/(+)-catechin; Ct-MGAM/chlorogenic acid; Nt-MGAM/chlorogenic acid; and Ct-SI/EGCG) out of the twenty different inhibitor-subunit combinations studied, showed the same values for Ki_1 and Ki_2. In these cases; therefore, the binding affinity of phenolic compounds to the free enzyme and enzyme-substrate complex was essentially the same, indicating that there was a single binding site for phenolics, and the binding of substrate did not interfere with the binding of phenolics at their binding site. This also implies the occurrence of conformational changes in the enzyme after binding of these phenolics, with effect on their substrate binding capability or catalytic mechanism. Results of another study using rat intestinal acetone powder were in accordance with our results, where chlorogenic acid showed a non-competitive inhibition behavior on maltase and sucrose activities (Ishikawa et al., 2007). However, in that study the IC_{50} values of chlorogenic acid for maltase and sucrase were 2.99 and 2.18 mM, which is substantially higher than our calculated IC_{50} values (Table 2) (Ishikawa et al., 2007). This difference could be explained by interference by multiple protein components present in the crude preparations of rat mucosal proteins. In another study, EGCG was shown as a competitive inhibitor and its IC_{50} was estimated as 20 μM for human intestinal maltase (Nguyen et al., 2012). In our study, caffeic and gallic acid were

not as strong inhibitors as chlorogenic acid and EGCG. Results of an earlier study showed that caffeic acid had very low inhibitory effect with IC_{50} values of 0.74 and 0.49 mM for maltase and sucrase, respectively (Welsch et al., 1989).

Stability of phenolic compounds must be considered, for such a selective inhibition concept to be pursued in a practical sense. In one study, chlorogenic acid and caffeic acid were found to be stable after incubating with human gastric juice, duodenal fluid and ileostomy effluent (Olthof et al., 2001). Another study showed that most (97.9-99.2%) of orally administered chlorogenic acid (700 µmol/kg) and a small amount of caffeic acid (0.8-2.1%) were found in the rat small intestine (Azuma et al., 2000). Record and Lane (2001) tested the stability of gallic acid, caffeine, and EGCG in green tea at acidic pH and at a slightly alkaline pH. Caffeine concentration did not change under the exposure of either acidic (pH 2.0) or slightly alkaline pH (pH 7.5) conditions. Also, the concentration of gallic acid was only slightly reduced at pH of 2.0 and at pH of 7.5. On the other hand, although EGCG concentration slightly declined at pH of 2.0; its concentration was lower with incubation for 1 h at pH of 7.5, decreasing from 1880 to 21 µmol/l in 1 g of green tea/50 ml of water. Several studies also reported that while acidic pH similar to that of the stomach slightly affected the concentration of catechins in green and black tea, the slightly alkaline pH (above pH 7.4) caused a significant decrease in their concentration (Green et al., 2007; Tenore et al., 2015; Zhu et al., 1997; Yoshino et al., 1999). Those studies used alkaline pH (above pH 7.4); however, it may not reflect the actual pH of the lumen of the entire small intestine (pH measured in humans subjects was found to be 6.4-6.6 in the duodenum or proximal small intestine and increased to 7.3-7.5 in the distal small intestine) (Fallingborg et al., 1989; Evans et al., 1988). This difference

could well be important as the glycemic spike seen in the response profile comes from rapid proximal digestion of glycemic carbohydrates, and at this lower pH the catechins could still be in high qualities with inhibition potential.

In conclusion, the finding of selective inhibition by phenolics of the individual mucosal α-glucosidases implies that digestion rate of starch, starch products, and sucrose can be modulated by these dietary components. The potential to control glycemic response profiles or sucrose hydrolysis rate using this selective inhibition concept with phenolic compounds warrants further study and as well using an in vivo model.

3.6   References

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Table 3.1 Inhibition mechanism and inhibition constant (Ki) for each α-glucosidase subunit.

	Inhibition mechanism and inhibition constant (Ki) ($\mu M \pm sd$)			
	Ct-MGAM	**Nt-MGAM**	**Ct-SI**	**Nt-SI**
Caffeic acid	Non-competitive heterogeneous Ki_1: 56.5 ± 25.4 Ki_2: 191.8 ± 39.6 r^2= 0.918	Non-competitive heterogeneous Ki_1: 75.2 ± 13.3 Ki_2: 478.3 ± 167.1 r^2=0.968	Non-competitive heterogeneous Ki_1: 58.2 ± 21.2 Ki_2: 323 ± 100.9 r^2= 0.902	Non-competitive homogenous Ki: 103.3 ± 11.1 r^2= 0.909
Gallic acid	Non-competitive heterogeneous Ki_1: 24.9 ± 8.6 Ki_2: 50 ± 5.7 r^2=0.978	Non-competitive heterogeneous Ki_1: 85.7 ± 13.9 Ki_2: 460.6 ± 133.2 r^2= 0.971	Non-competitive heterogeneous Ki_1: 31.3 ± 7.2 Ki_2: 142.8 ± 24.7 r^2=0.969	Non-competitive heterogeneous Ki_1: 44.6 ± 14.4 Ki_2: 127.6 ± 28.7 r^2=0.93
(+)-Catechin	Non-competitive heterogeneous Ki_1: 20.4 ± 8.2 Ki_2: 96.7 ± 21.7 r^2=0.925	Non-competitive heterogeneous Ki_1: 20.4 ± 3.8 Ki_2: 97.2 ± 26.9 r^2= 0.967	Non-competitive heterogeneous Ki_1: 21.6 ± 8.1 Ki_2: 45.4 ± 5.9 r^2=0.973	Non-competitive homogenous Ki: 33.7 ± 4.1 r^2= 0.921
Chlorogenic acid	Non-competitive homogenous Ki: 3 ± 0.3 r^2=0.937	Non-competitive homogenous Ki: 43.2 ± 3.5 r^2=0.943	Non-competitive heterogeneous Ki_1: 1.8 ± 0.3 Ki_2: 28 ± 8.8 r^2=0.983	Non-competitive heterogeneous Ki_1: 101.8 ± 36.6 Ki_2: 259.7 ± 114.5 r^2=0.93
EGCG	Non-competitive heterogeneous Ki_1: 1.7 ± 0.7 Ki_2: 6.5 ± 2.1 r^2=0.941	Non-competitive heterogeneous Ki_1: 9 ± 1.5 Ki_2: 97.6 ± 49.3 r^2=0.967	Non-competitive homogenous Ki: 15.1 ± 1.6 r^2=0.939	Non-competitive heterogeneous Ki_1: 76.3 ± 31.5 Ki_2: 140.4 ± 53.5 r^2=0.919

Table 3.2 IC$_{50}$ values for each α-glucosidase subunit.

	IC$_{50}$ (95% CI) (µM)			
	Ct-MGAM	**Nt-MGAM**	**Ct-SI**	**Nt-SI**
Caffeic acid	191.4 (172.6, 212.3)	266 (215.7, 327.9)	200.8 (177.3, 227.2)	137.4 (129.2, 146.2)
Gallic acid	60.9 (51.4, 72.1)	248.7 (217, 285)	129.2 (113.9, 146.6)	82.5 (60.8, 111.9)
(+)-Catechin	79.7 (57.9, 109.6)	55.5 (50.2, 61.3)	34.3 (15.4, 76.6)	36 (32.3, 40.1)
Chlorogenic acid	2.3 (1.3, 3.9)	60 (50.8, 70.8)	13.2 (11.6, 15.1)	159.4 (137.4, 184.9)
EGCG	3.5 (2.4, 5.1)	39.6 (34.3, 45.8)	13.8 (11.4, 16.7)	126 (92.2, 172.1)

A

$$E + S \leftrightarrow ES \rightarrow E + P$$
$$+ I$$
$$Ki \updownarrow$$
$$EI$$

B

$$E + S \leftrightarrow ES \rightarrow E + P$$
$$+ I$$
$$Ki \updownarrow$$
$$ESI$$

C

$$E + S \leftrightarrow ES \rightarrow E + P$$
$$+ I \qquad + I$$
$$Ki \updownarrow \qquad \updownarrow Ki$$
$$EI \qquad ESI$$

D

$$E + S \leftrightarrow ES \rightarrow E + P$$
$$+ I \qquad + I$$
$$Ki_1 \updownarrow \qquad \updownarrow Ki_2$$
$$EI \qquad ESI$$
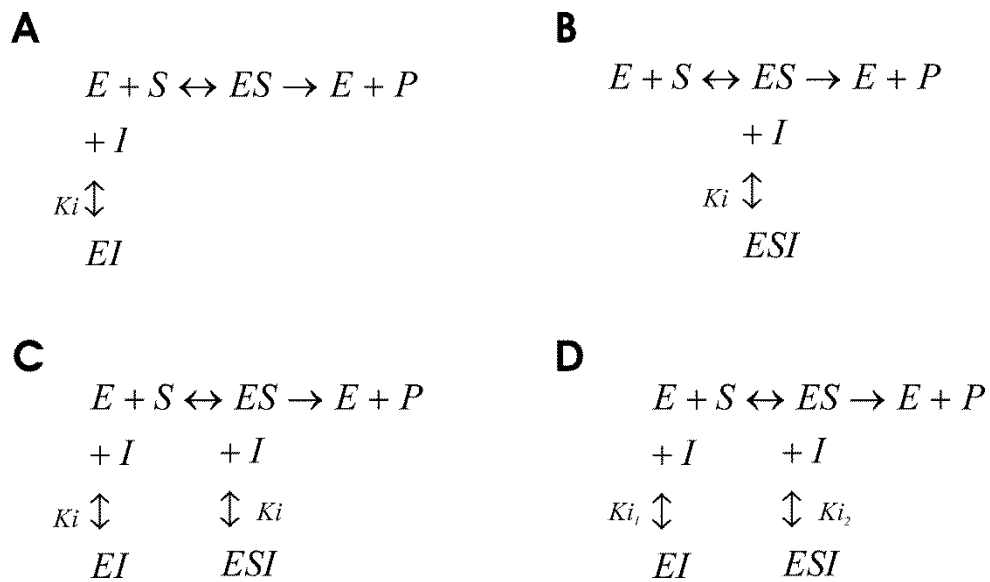
Figure 3.1 The assumed reaction mechanisms for each of the different inhibition types. (A) The competitive inhibition. (B) The uncompetitive inhibition. (C) Non-competitive homogeneous inhibition. (D) Non-competitive mixed heterogeneous inhibition.
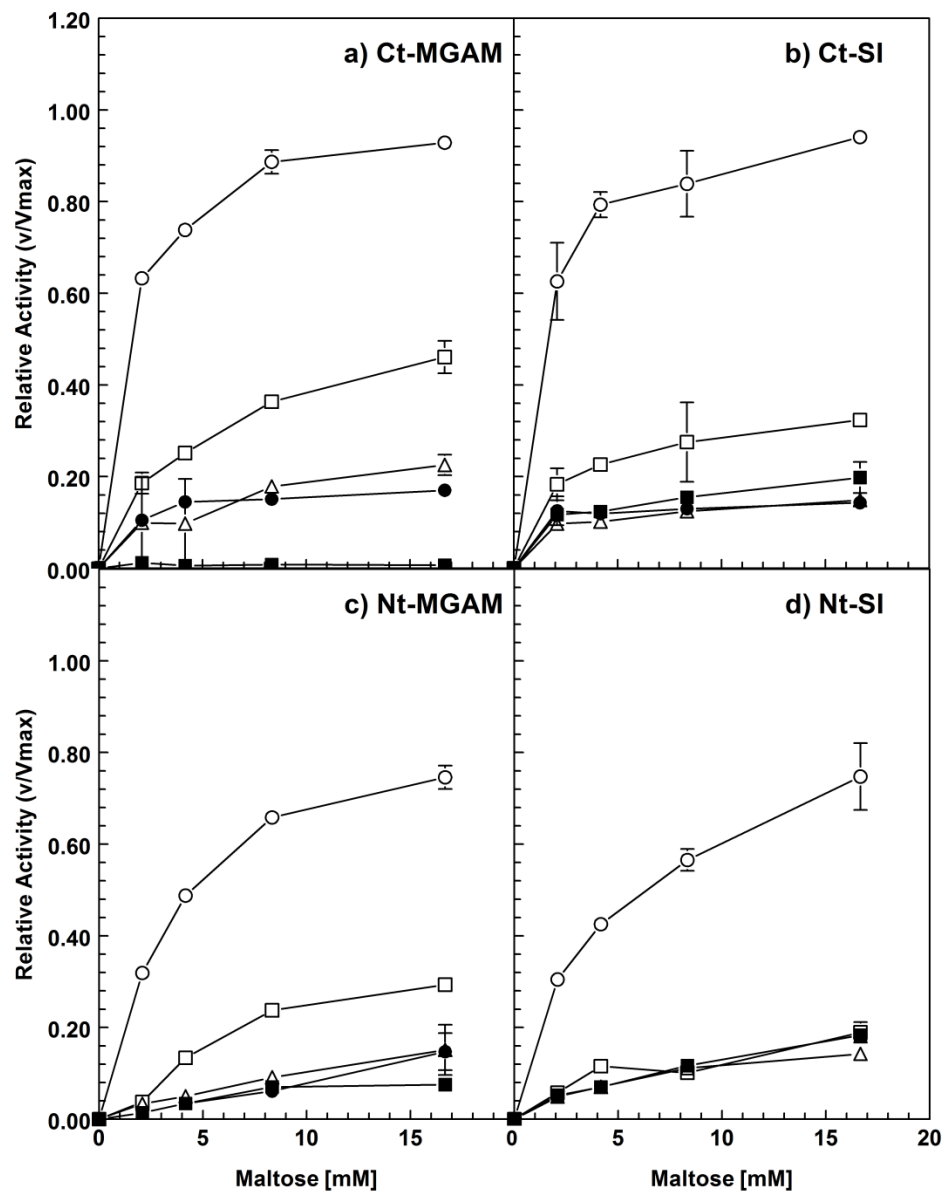
Figure 3.2 Relative activity (v/Vmax) change versus different maltose concentrations (2.1,4.2, 8.3 and 16.7 mM) for: a) Ct-MGAM, b) Ct-SI, c) Nt-MGAM, and d) Nt-SI in the presence of (+)-catechin at (○) 0 μM, (□) 83.3 μM, (Δ) 166.7 μM, (●) 333.3μM and (■) 666.7 μM.
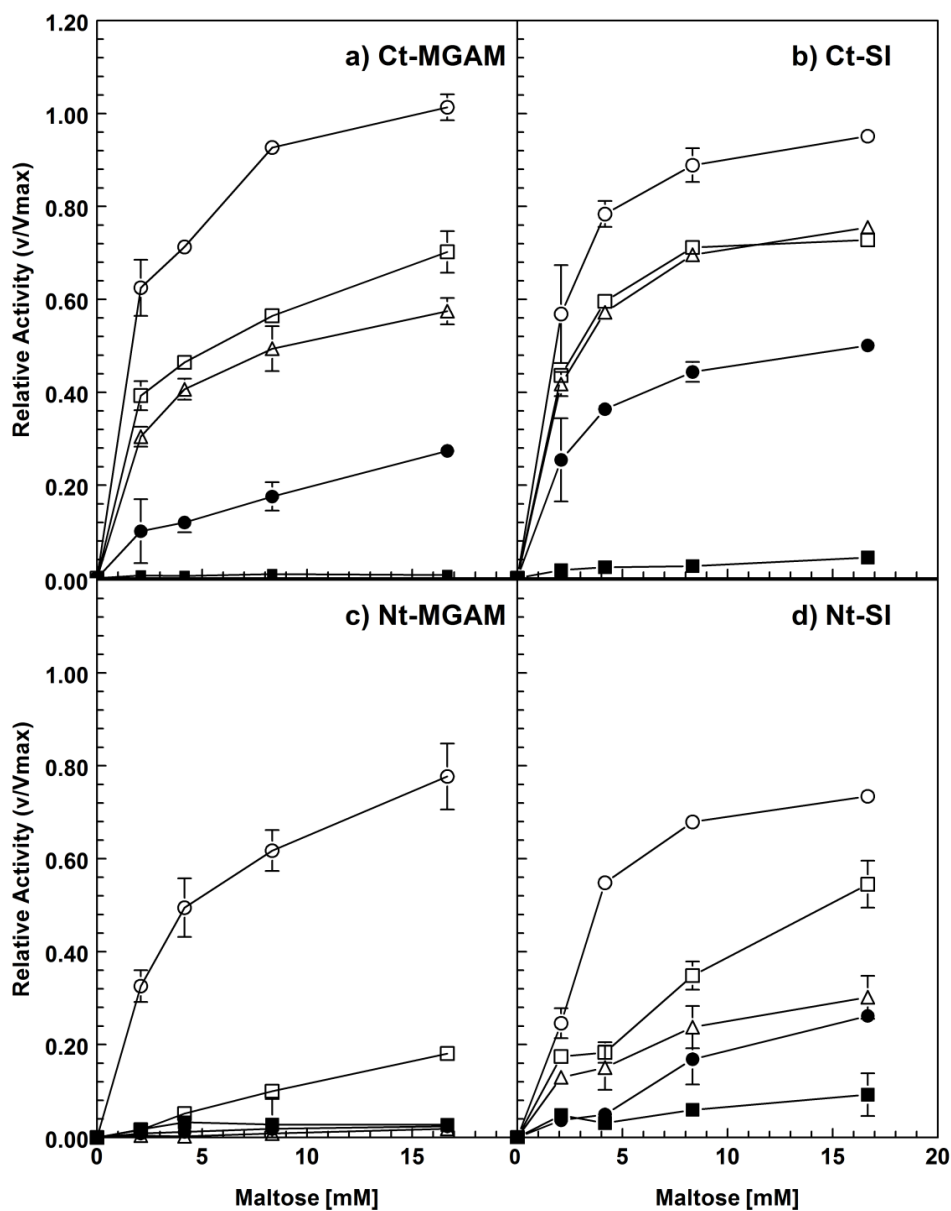
Figure 3.3 Relative activity (v/Vmax) change versus different maltose concentrations (2.1, 4.2, 8.3 and 16.7 mM) for: a) Ct-MGAM, b) Ct-SI, c) Nt-MGAM and d) Nt-SI in the presence of EGCG at (○) 0 μM, (□) 0.67 μM, (Δ) 3.3 μM, (●) 16.7 μM and (■) 83.3 μM for Ct-MGAM and Ct-SI and at (○) 0 μM, (□) 83.3 μM, (Δ) 166.7 μM, (●) 333.3 μM and (■) 666.7 μM for Nt-MGAM and Nt-SI.
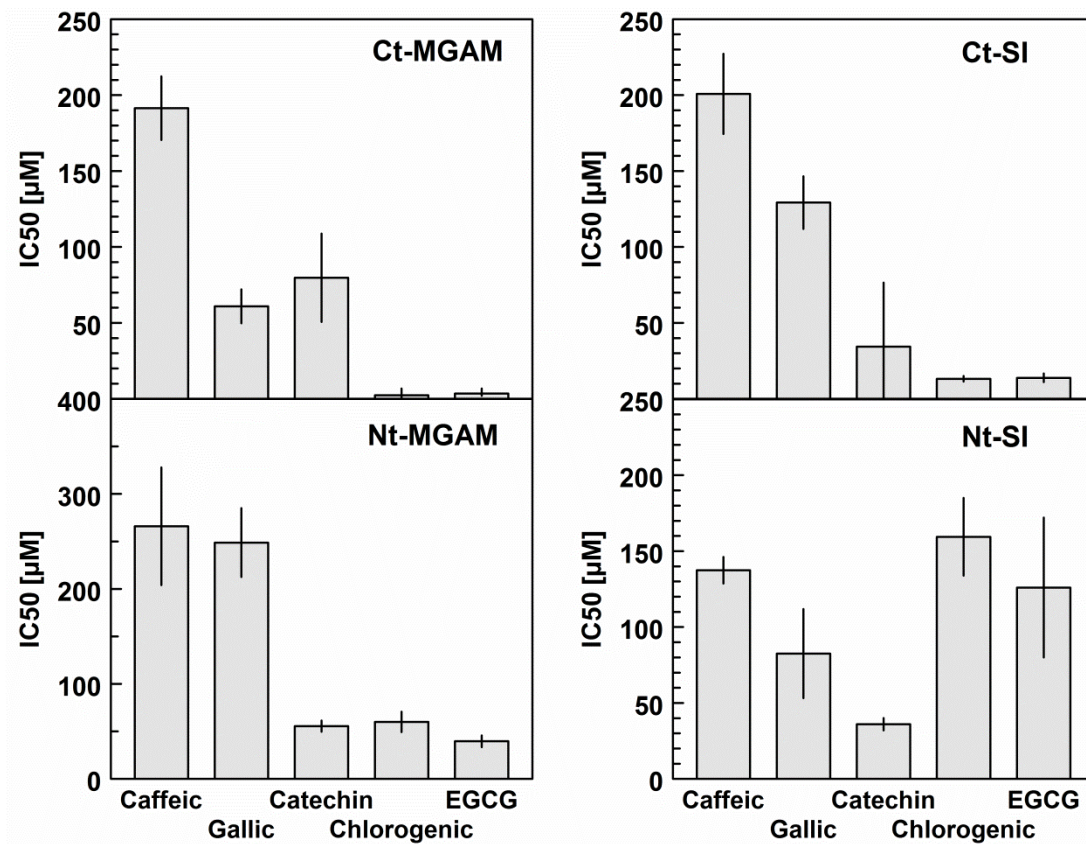
Figure 3.4 IC_{50} values (µM) of caffeic acid, gallic acid, (+)-catechin, chlorogenic acid, and EGCG for Ct-MGAM, Nt-MGAM, Ct-SI and Nt-SI. The vertical lines represent the 95% CI for the calculated values.

CHAPTER 4. DIETARY PHENOLIC COMPOUNDS HAVE DIFFERENT INHIBITORY POTENCIES ON THE MUCOSAL ALPHA-GLUCOSIDASES OF RAT INTESTINAL ACETONE POWDER TO REGULATE THE GLUCOSE RELEASE

4.1   Abstract

Certain plant phenolic acids and polyphenols have been recognized to have inhibitory effect on the enzymatic activities of intestinal α-glucosidases. The objective of this study was to determine the inhibitory capacities of selected phenolic compounds for the inhibition of maltase, sucrase and isomaltase activities in rat intestinal acetone powder. Inhibition mechanisms, inhibitory constants, as well IC_{50} values were determined for maltase, sucrase, and isomaltase activities using gallic acid, caffeic acid, (+)-catechin, chlorogenic acid, and (-)- epigallocatechin gallate (EGCG). The Tris glucose-oxidase peroxidase method was performed to measure glucose release. Except chlorogenic acid, all compounds inhibited maltase, sucrase, and isomaltase activities in a non-competitive homogeneous manner. Among the compounds, EGCG and chlorogenic acid displayed the highest inhibitory potency for maltase, sucrase, and isomaltase. Particularly, polyphenol inhibition of maltase and sucrase activities is promising for modulation of the rate of glucose release in the small intestine for the improvement of glycemic response.

Keywords: inhibition, maltase, phenolic compounds, sucrase, α-glucosidases.

4.2 Introduction

Starchy foods carry a vital importance in the human diet, because they provide required energy to the body. Starch digestion into glucose requires the salivary and pancreatic α-amylases and α-glucosidases. First, digestion of starch by the α-1,4-endoglucosidases, salivary and pancreatic α-amylases, generates linear glucose oligomers, branched α-limit dextrins, and very little free glucose (Quezada-Calvillo et al., 1993). Then, the mucosal α-glucosidases, Maltase-Glucoamylase (MGAM) and Sucrase-Isomaltase (SI), located on the small intestinal brush border membrane, convert α-amylase degradation products into free glucose (Quezada-Calvillo et al., 2007). It has been reported that all individual subunits of MGAM and SI have maltase activity (Jones et al., 2011; Auricchio et al., 1965). However, there are some specific substrates which help to differentiate the activities of the four subunits. For instance, sucrase and isomaltase activities cleave the α-1-2 linkage of sucrose and α-1-6 linkage of isomaltose (Sim et al., 2008). Since all of these activities result in the formation of free glucose in the small intestine, which is absorbed and circulated into the blood stream (Quezada-Calvillo et al., 1993), the inhibition of these activities modifies the rate of its release. Such inhibition may confer various health benefits, such as improving insulin sensitivity and the glycemic response profile in Type 2 diabetics or pre-diabetics. Although, there are known strong inhibitors of α-glucosidases, such as acarbose (Renet al., 2011), side effects are common such as diarrhea or abdominal discomfort resulting from large amounts of undigested starch or α-amylase degradation products reaching the colon. Therefore, numerous studies have tested the inhibitory potential of different chemically

synthesized compounds (Eskandari et al., 2010; Rossi et al., 2006), and extracts obtained from natural sources such as plants (Adisakwattana et al., 2012; He et al., 2013; Mata et al., 2013) . Phenolic is one group which has received significant attention due to their natural source and their previously identified inhibitory effects on the activities of α-glucosidases (Ramkumar et al., 2010; Ishikawa et al. 2007; McDougall et al., 2008). For example, the inhibitory effect of gallic acid was shown on rat sucrase and maltase activities (Gupta et al., 2007). It was also reported that the gallic acid, (+)- catechin, and (-)- epigallocatechin gallate significantly inhibited the relative activity of rat small intestinal sucrase and α-glucosidase (Honda et al., 1993). Another study showed that EGCG from different teas inhibited the α-glucosidase activity of rat intestinal acetone powder (Xu et al., 2013; Koh et al., 2010). Caffeic acid and chlorogenic acid were also shown as potent inhibitors of rat intestinal α-glucosidase (Ishikawa et al., 2007; Adisakwattana et al., 2009).

Thus, phenolic compounds are promising candidates to explore in way of their selective or differential inhibition of the individual α-glucosidases in order to understand how to more precisely regulate the rate of digestibility of starchy foods.

Here, our study focused on investigating the selective or differential inhibitory effects of phenolic compounds gallic acid, caffeic acid, (+)-catechin, chlorogenic acid and EGCG, on maltase, sucrase and isomaltase activities of α-glucosidases present in the rat intestinal acetone powder. We determined the inhibition mechanisms and the respective inhibition constants for each of these activities to understand the sensitivity of different activities of rat intestinal acetone powder to the tested polyphenolic compounds.

4.3 <u>Materials and Methods</u>

4.3.1 Materials

Intestinal acetone powder from rat (Sigma-Aldrich, St. Louis, MO, USA) was used as a source of α-glucosidases for activity and inhibition assays. D-(+)-maltose monohydrate, sucrose, isomaltose, and D-lactose monohydrate were purchased from Sigma-Aldrich. Gallic acid, caffeic acid, chlorogenic acid, (+)-catechin and (-) epigallocatechin gallate (EGCG) were also obtained from Sigma-Aldrich.

4.3.2 Protein determination of rat intestinal acetone powder

The Bio-Rad Protein Assay Kit (Hercules, CA, USA) was used to determine the protein amount in rat intestinal acetone powder. Briefly, 100 mg of rat intestinal acetone powder was vortexed in 5 ml of 10 mM PBS-EDTA (pH=6.8) solution for 30 min at 4 °C. The supernatant was collected after the centrifugation (10,000 g x 30 min) at 4 °C. Ten µl of diluted 20 mg/ ml of the rat intestinal acetone powder solution and 200 µl of dye reagent were mixed and incubated at a room temperature for at least 5 min. Serial dilutions of stock solution of 0.5 mg/ ml of bovine serum albumin were used as standards. The absorbance values were read at 595 nm using a Synergy HT microplate reader (BioTek; Winooski, VT, USA).

4.3.3 Enzyme activity determination of disaccharidases of rat intestinal acetone powder

Ten µl of 20 mg/ml of rat intestine acetone powder enzyme solution was reacted with 10 µl of D-(+)-maltose monohydrate, sucrose, isomaltose, and lactose at a final

concentration range of 3.125-25 mM, and dissolved in 0.1 M maleate buffer in a 96 well plate for 1 h at 37 °C (Quezada-Calvillo et al., 1993; Dahlqvist et al., 1964). Maltose, sucrose, and isomaltose solutions were prepared at pH 6.4, while lactose solution was at pH 5.6. The reaction was stopped by the addition of 180 µl of Tris glucose oxidase (TGO) reagent and the amount of glucose release was measured at 450 nm after 45 min additional incubation in the microplate reader. The Michealis-Menten constant (Km) and maximal specific activity (mU/mg protein) for each specific substrate were determined. The possible interaction of phenolics with the intermediates generated through the glucose oxidase peroxidase reaction was corrected for as described in the Section 3.3.4 of previous chapter.

4.3.4   Inhibition assays of disaccharidases of rat intestinal acetone powder

Ten µl of the inhibitor at a final concentration range of 0-666.7 µM was reacted with 10 µl of four final different concentrations of maltose, sucrose and isomaltose (2.1, 4.2, 8.3 and 16.7 mM) in the presence of 10 µl of enzyme solution for the inhibition of maltase, sucrase, and isomaltase activities at 37 °C in triplicate. The reaction was stopped by adding 170 µl of TGO reagent and the mixture was incubated for 45 min. The optical density was measured at 450 nm. The inhibition mechanisms and inhibition constants (Ki) were calculated for each inhibitor using data points obtained in triplicate.

4.3.5   Determination of inhibition mechanism and inhibition constant

Nonlinear regression analysis using the Levenberg-Marquardt algorithm was performed applying the standard equations of competitive, uncompetitive, non-

competitive homogeneous, and non-competitive heterogeneous inhibition using Sigma Plot, (Systat Software Inc.; San Jose, CA., USA). The inhibition mechanism and the respective Ki value were decided with the best mathematical fit for the enzyme-inhibitor combination. The significance value (p value) for the statistical analysis was chosen as less than 0.05.

The used inhibition mechanisms in this study are given in Figure 4.1. When the inhibitor binds to the active site of the enzyme with a specific Ki value, this is termed competitive inhibition. In uncompetitive inhibition, the inhibitor binds the enzyme-substrate complex with a specific Ki value. In case of non-competitive inhibition, the inhibitor binds to the substrate as well the enzyme-substrate intermediate product. When the specific Ki values are the same for this type of binding, this is known as non-competitive homogeneous inhibition. Non-competitive heterogeneous inhibition occurs when different specific Ki values are present for the substrate and enzyme-substrate complex. The equations for the inhibition mechanisms are given below where the rate of reaction is 'v', the calculated maximal rate of reaction is Vmax, substrate concentration is [S], the calculated Michealis constant is 'Km', the inhibitor concentration is [I], and the inhibition constant is 'Ki'.

Eqn. 1. Competitive Inhibition:

$$v = \frac{Vmax * [S]}{Km * \left(1 + \frac{[I]}{Ki}\right) + [S]}$$

Eqn. 2. Uncompetitive Inhibition:

$$v = \frac{Vmax * [S]}{Km + [S] * \left(1 + \frac{[I]}{Ki}\right)}$$

Eqn. 3. Non-competitive homogeneous inhibition

$$v = \frac{Vmax * [S]}{(Km + [S]) * (1 + \frac{[I]}{Ki})}$$

Eqn. 4. Non-competitive heterogeneous inhibition

$$v = \frac{Vmax * [S]}{Km * \left(1 + \frac{[I]}{Ki1}\right) + [S] * \left(1 + \frac{[I]}{Ki2}\right)}$$

4.3.6   Determination of IC_{50} values

IC_{50} value was defined as the concentration of each inhibitor needed for inhibiting 50% of the maltase, sucrase and isomaltase activities of rat intestinal acetone powder solution. It was determined by linear regression of the log transformed values of inhibitor concentration (log [I]) versus the relative activity (v/Vmax) at 16.7 mM maltose, sucrose, and isomaltose concentration, using Minitab 14 (State College, Pennsylvania, USA).

4.4   Results

The disaccharidase (maltase, sucrase, isomaltase, and lactase) activities of rat intestinal acetone powder solution were measured. The average maximal specific activities of disaccharidases of rat intestinal acetone powder solution were 462.85, 85.8, 124.96, and 15.17 mU/mg protein for maltase, sucrase, isomaltase, and lactase (Table

4.1). Km values for maltase, sucrase, isomaltase, and lactase activities of rat intestinal acetone powder solution were 1.44, 16.00, 19.04, and 14.94 mM, respectively (Table 4.1).

4.4.1 The mechanism and constants for the inhibition of maltase, sucrase and isomaltase activities

Kinetic analysis was carried out to understand the inhibitory characteristics of gallic acid, caffeic acid, (+)-catechin, chlorogenic acid, and EGCG. The inhibition mechanisms and inhibition constants for the maltase, sucrase, and isomaltase activities of rat intestinal acetone powder were determined. Caffeic and gallic acid showed a non-competitive homogeneous inhibition type for maltase activity of rat intestinal acetone powder with a Ki value of 0.199 mM and 0.069 mM, respectively (Table 4.2, Figure 4.7 and 4.8). Sucrase and isomaltase activities of rat intestinal acetone powder were also inhibited by caffeic and gallic acid in a non-competitive homogeneous manner with Ki values of 0.589 and 0.152 and 0.861 and 0.166 mM, respectively (Table 4.2, Figure 4.2 and 4.3). Caffeic and gallic acid demonstrated a higher inhibitory potency towards maltase, than sucrase or isomaltase activities of rat intestinal acetone powder. Caffeic acid showed higher Ki values, or higher inhibitory potency, for all the activities than gallic acid. (+)-Catechin demonstrated non-competitive homogeneous inhibition with a Ki value of 0.069 mM for maltase activity of rat intestinal acetone powder (Table 4.2, Figure 4.9). Similar to that, it inhibited the sucrase and isomaltase activities of rat intestinal acetone powder in a non-competitive homogenous manner with Ki values of 0.093 and 0.098 μM, respectively (Table 4.2 and Figure 4.4). (+)-Catechin had only slightly higher inhibitory activity for maltase, than sucrase and isomaltase. Therefore, it

exhibited similar inhibition potency towards all tested activities of rat intestinal acetone powder. This compound and gallic acid showed the same relatively high inhibitory potency for maltase activity. Chlorogenic acid showed non-competitive homogeneous inhibition for maltase activity of rat intestinal acetone powder at Ki value of 0.056 mM (Table 4.2 and Figure 4.10). Here, sucrase activity was also inhibited competitively with a Ki value of 0.055 mM (Table 4.2 and Figure 4.5). The inhibitory potencies of chlorogenic acid for maltase and sucrase were approximately the same. As shown in Table 4.2 and Figure 4.5, chlorogenic acid inhibited isomaltase activity with a lower binding affinity (Ki=0.088 mM). EGCG demonstrated non-competitive homogenous inhibition for the maltase, sucrase, and isomaltase activities of the rat intestinal acetone powder. This was the strongest inhibiting polyphenol of those tested, and for all three activities, with inhibition constants (Ki) of 0.006 mM for maltase (Figure 4.11), 0.036 mM for sucrase (Figure 4.7), and 0.056 mM for isomaltase (Table 2 and Figure 4.7). The inhibitory potency of EGCG was approximately 6 to 8 times greater for the maltase activity compared to the sucrase and isomaltase activities of rat intestinal acetone powder.

These results showed that EGCG and chlorogenic acid had the highest inhibitory potencies [lowest inhibition constants (Ki)] for maltase, sucrase and isomaltase activities of rat intestinal acetone powder among the tested phenolics. Furthermore, selective inhibition of rat intestinal was shown for maltase rather than sucrase and isomaltase particularly for EGCG, chlorogenic acid, and caffeic acid. With the exception of chlorogenic acid showing the competitive inhibition type, all other tested compounds indicated non-competitive homogeneous inhibition for the maltase, sucrase, and isomaltase activities.

4.4.2 The IC_{50} values

IC_{50} values were determined for the inhibition of maltase, sucrase, and isomaltase activities of rat intestinal acetone powder. For the maltase inhibition, all tested compounds had IC_{50} values in the range of 0.011-0.195 mM (Table 4.3). This changed to 0.067-1.471 mM for the sucrase activity and to 0.043-1.962 mM for the isomaltase activity. Maltase activity inhibition required lower concentration of tested compounds for inhibition (lower IC_{50} values) than was found for sucrase and isomaltase activity inhibition, with the sole exception of (+)-catechin. According to the calculated IC_{50} values, and in concordance with Ki values, EGCG was the most potent inhibitor for all tested activities, particularly for maltase activity. Another compound presenting a clear preference towards maltase activity rather than sucrase and isomaltase activities, was caffeic acid, but with a lower inhibitory effect compared to EGCG.

4.5 Discussion and Conclusions

Phenolic compounds have been recognized as having inhibitory effect on the intestinal α-glucosidases, but it has not been shown what relative inhibitory effect they have on the individual hydrolytic activities of the combined enzymes (He et al., 2013; Mata et al., 2013; Ramkumar et al., 2010). Certainly a practical target is that they have the potential to be an effective dietary agent for controlling the rate of glucose release from starchy foods to improve diabetic condition, and conditions leading to obesity and pre-diabetes. The use of phenolic compounds can be more promising than the use of strong intestinal α-glucosidase inhibitors, such as acarbose, because their more mild

inhibition would allow starch to be digested slowly; thus, reducing abdominal discomfort and/or diarrhea that can occur due to the presence of large amounts of non-digested glycemic carbohydrates in the colon, and perhaps slow digestion would result in beneficial gut hormone triggers in the distal small intestine.

Although various studies were reported to show the inhibitory effect of different phenolic compounds on α-glucosidases of rat intestinal acetone powder, here we introduce the concept of the possible differential and selective inhibition of different overall specific activities of α-glucosidases in rat intestinal acetone powder. For instance, according to the calculated Ki values, all tested compounds except chlorogenic acid presented a preference for the rat intestinal maltase, rather than sucrase and isomaltase. Furthermore, EGCG and chlorogenic acid were the most potent inhibitors for combined intestinal maltase and sucrase among all tested compounds, and here sucrase inhibition looks like a possible beneficial outcome. EGCG was shown by others to inhibit α-glucosidase of rat intestinal acetone powder with an IC_{50} of 914.8 μmol/L (Xu et al., 2013), although this is considerably higher than our calculated IC_{50}'s (11-67 μM) for maltase, sucrase, or isomaltase. Results of another earlier study (Ishikawa et al., 2007) showed chlorogenic acid as a non-competitive inhibitor for rat intestinal maltase and sucrase with IC_{50} values of 2.99 and 2.18 mM, but this was also much higher than our calculated IC_{50} values. Also, Matsui et al. (2004) showed high IC_{50} values (18.9 and 3.1 mM) of chlorogenic acid for maltase and sucrase activities of rat intestinal acetone powder, respectively using immobilized enzyme system instead of using free enzyme system. These variations of our results from previous reported ones may be due to some differences in performed inhibition assays, such as use of a heating step to stop enzymatic

reaction (Ishikawa et al., 2007), using different substrate (p-nitrophenyl α-D-glucopyranoside) to determine the glucose release, or using an immobilized enzyme system than the free enzyme system. The reason of obtaining higher IC_{50} values using immobilized enzyme system may be restricting binding of the compound to the enzyme. Modifications in inhibition assay can affect the final Ki and IC_{50} values significantly. In contrast to EGCG and chlorogenic acid, caffeic acid showed less inhibitory potency. Caffeic acid was also found as a weak inhibitor for maltase and sucrase with IC_{50} values of 0.74 and 0.49 mM, respectively in a previous study (Welsh et al., 1989).

In Chapter 3, EGCG and chlorogenic acid were found to be the most efficient inhibitors for the inhibition of glucoamylase subunit. Similar to that, in this study, they are compounds showing higher inhibitory potencies towards maltase, sucrase and isomaltase activities of rat intestinal acetone powder. The current study provides a view of the overall effect of tested compounds on the specific activities of enzyme mixture of MGAM and SI in the rat intestinal acetone powder, rather than on the individual enzyme subunits.

In Chapter 3, we found that lower Ki and IC_{50} were associated with the inhibition of individual subunits of MGAM and SI than for the inhibition of maltase and sucrase of rat intestinal acetone powder. These results are expected since the subunits can share activities, particularly that of maltase. Also, there can be possible non-specific binding to other proteins present in the rat intestinal acetone powder which might result in higher Ki values. We did not take the non-specific binding into account in this study, but this may be further studied whether there is an interaction of tested phenolics with other proteins locating on the brush border membrane.

Our results suggest that with the use of polyphenol compounds there is a capacity to generate distinct rates or profiles of digestion of starches, starch products, and sucrose. For instance, because maltase is a fasting acting activity and isomaltase a slow one, combining highly branched starchy products with EGCG or chlorogenic acid should cause slowing of the rate of glucose release in the intestine due to their high inhibitory effect on maltase activity and relatively little effect on isomaltase activity. In this way a complete digestion of starch can still be obtained, as the low inhibitory effect on isomaltase activity still allows digestion starch branch points effectively. Additionally, there is the potential that slowly digestible glycemic carbohydrates, whether they are starch or sucrose, could activate the ileal brake and gut-brain axis mechanisms in order to slow gastric emptying and control appetite for weight management (Lee et al., 2013).

In conclusion, the demonstrated differential inhibition of the α-glucosidase activities of rat intestinal acetone powder shows that there is a potential to modulate the rate of digestion of starchy, or sucrose containing, foods in the presence of specific phenolic compounds.

4.6   <u>References</u>

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Table 4.1 Maltase, sucrase, isomaltase, and lactase activities of rat intestinal acetone powder.

	Km (mM) ± sd	Maximal specific activity (mU/mg protein)
Maltase	1.44 ± 0.09	462.85
Sucrase	16.00 ± 0.76	85.8
Isomaltase	19.04 ± 2.84	124.96
Lactase	14.94 ± 1.01	15.17

Table 4.2 Inhibition mechanisms and inhibition constants for maltase, sucrase, and isomaltase activities of rat intestinal acetone powder for caffeic acid, gallic acid, (+)-catechin, chlorogenic acid, and EGCG.

	Maltase activity inhibition $K_i \pm sd$ (mM)	Sucrase activity inhibition $K_i \pm sd$ (mM)	Isomaltase activity inhibition $K_i \pm sd$ (mM)
Caffeic acid	Non-competitive homogeneous $K_i= 0.199 \pm 0.023$ $r^2= 0.90$	Non-competitive homogeneous $K_i= 0.589 \pm 0.092$ $r^2= 0.92$	Non-competitive homogeneous $K_i= 0.861 \pm 0.178$ $r^2= 0.90$
Gallic acid	Non-competitive homogeneous $K_i= 0.069 \pm 0.008$ $r^2= 0.90$	Non-competitive homogenous $K_i= 0.152 \pm 0.017$ $r^2= 0.93$	Non-competitive homogeneous $K_i= 0.166 \pm 0.019$ $r^2= 0.92$
(+)-Catechin	Non-competitive homogeneous $K_i= 0.069 \pm 0.008$ $r^2= 0.90$	Non-competitive homogeneous $K_i= 0.093 \pm 0.012$ $r^2= 0.90$	Non-competitive homogeneous $K_i= 0.098 \pm 0.013$ $r^2= 0.90$
Chlorogenic acid	Non-competitive homogeneous $K_i= 0.056 \pm 0.009$ $r^2= 0.90$	Competitive $K_i= 0.055 \pm 0.007$ $r^2= 0.95$	Non-competitive homogeneous $K_i= 0.088 \pm 0.011$ $r^2= 0.90$
EGCG	Non-competitive homogeneous $K_i= 0.006 \pm 0.0004$ $r^2= 0.97$	Non-competitive homogeneous $K_i= 0.036 \pm 0.003$ $r^2= 0.95$	Non-competitive homogeneous $K_i= 0.056 \pm 0.007$ $r^2= 0.93$

Table 4.3 IC50 values for the inhibition of maltase, sucrase, and isomaltase activities of rat intestinal acetone powder for gallic acid, caffeic acid, (+)-catechin, chlorogenic acid, and EGCG.

	Maltase activity inhibition IC$_{50}$ (mM) ± sd	Sucrase activity inhibition IC$_{50}$ (mM) ± sd	Isomaltase activity inhibition IC$_{50}$ (mM) ± sd
Gallic acid	0.109 ± 0.016	0.193 ± 0.031	0.201 ± 0.023
Caffeic acid	0.195 ± 0.023	1.471 ± 0.224	1.962 ± 0.314
(+)-Catechin	0.167 ± 0.022	0.115 ± 0.014	0.125 ± 0.018
Chlorogenic acid	0.116 ± 0.019	0.140 ± 0.023	0.152 ± 0.020
EGCG	0.011 ± 0.001	0.067 ± 0.007	0.043 ± 0.005

A

$$E + S \leftrightarrow ES \to E + P$$
$$+ I$$
$$Ki \updownarrow$$
$$EI$$

B

$$E + S \leftrightarrow ES \to E + P$$
$$+ I$$
$$Ki \updownarrow$$
$$ESI$$

C

$$E + S \leftrightarrow ES \to E + P$$
$$+ I \qquad + I$$
$$Ki \updownarrow \qquad \updownarrow Ki$$
$$EI \qquad ESI$$

D

$$E + S \leftrightarrow ES \to E + P$$
$$+ I \qquad + I$$
$$Ki_1 \updownarrow \qquad \updownarrow Ki_2$$
$$EI \qquad ESI$$
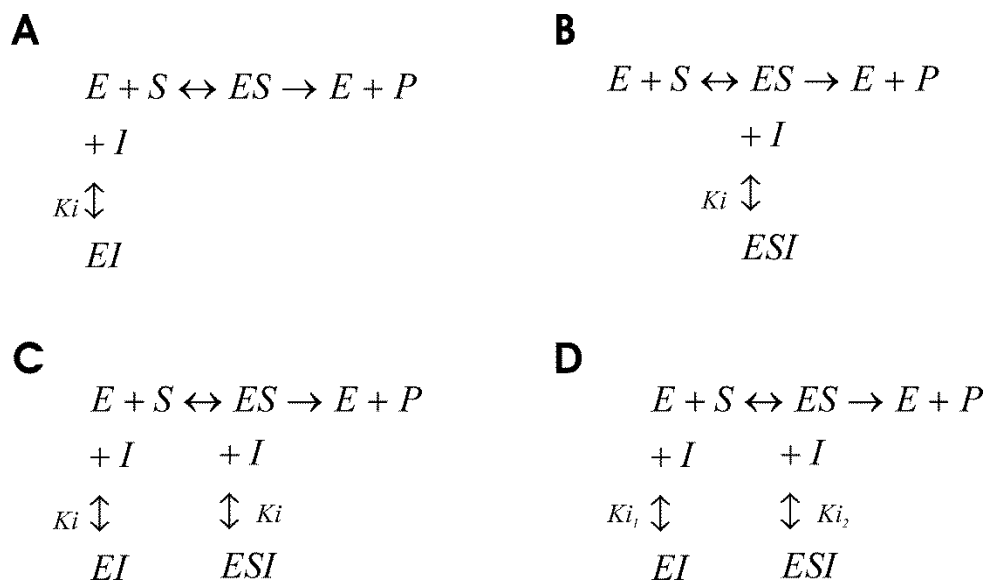
Figure 4.1 The assumed reaction mechanisms for each of the different inhibition types: (A) competitive inhibition, (B) uncompetitive inhibition, (C) non-competitive homogeneous inhibition, (D) non-competitive mixed heterogeneous inhibition.

Figure 4.2 Relative activity (v/Vmax) change versus different maltose concentrations (2.1, 4.2, 8.3 and 16.7 mM) for rat intestinal sucrase and isomaltase in the presence of caffeic acid at (○) 0 μM, (□) 83.3 μM, (Δ) 166.7 μM, (●) 333.3 μM and (■) 666.7 μM.

Figure 4.3 Relative activity (v/Vmax) change versus different maltose concentrations (2.1, 4.2, 8.3 and 16.7 mM) for rat intestinal sucrase and isomaltase in the presence of gallic acid at (○) 0 µM, (□) 83.3 µM, (Δ) 166.7 µM and (●) 333.3 µM.

Figure 4.4 Relative activity (v/Vmax) change versus different maltose concentrations (2.1, 4.2, 8.3 and 16.7 mM) for rat intestinal sucrase and isomaltase in the presence of (+)-catechin at (○) 0 µM, (□) 83.3 µM, (Δ) 166.7 µM and (●) 333.3 µM.

Figure 4.5 Relative activity (v/Vmax) change versus different maltose concentrations (2.1, 4.2, 8.3 and 16.7 mM) for rat intestinal sucrase and isomaltase in the presence of chlorogenic acid at (○) 0 µM, (□) 83.3 µM, (Δ) 166.7 µM and (●) 333.3 µM.

Figure 4.6 Relative activity (v/Vmax) change versus different maltose concentrations (2.1, 4.2, 8.3 and 16.7 mM) for rat intestinal sucrase and isomaltase in the presence of EGCG at (○) 0 µM, (□) 10.4 µM, (Δ) 20.8 µM, (●) 41.7 µM and (■) 83.3 µM.
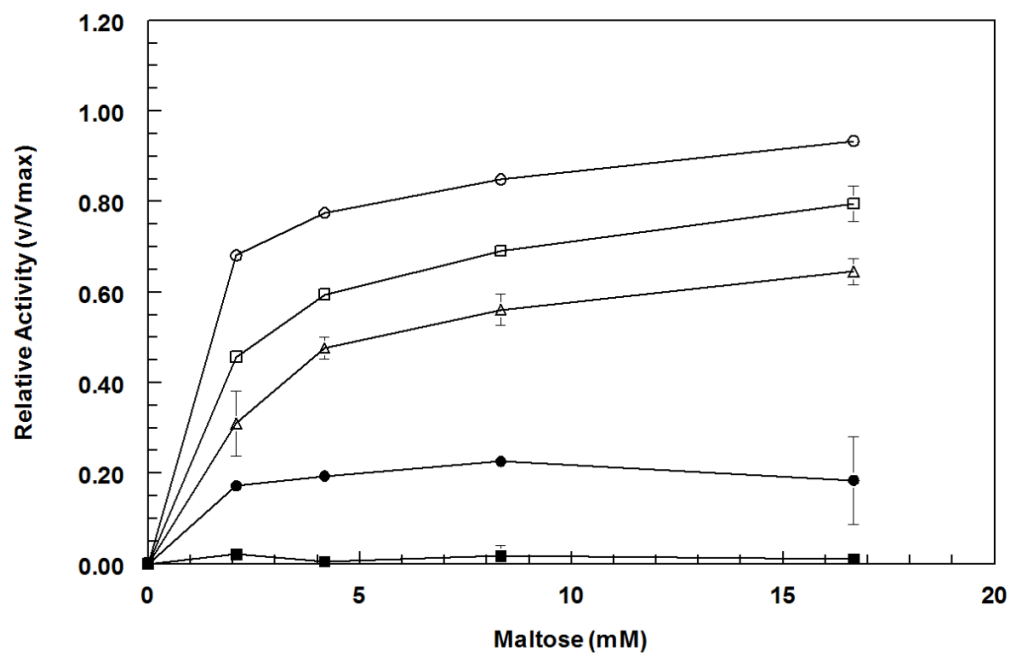
Figure 4.7 Relative activity (v/Vmax) change versus different maltose concentrations (2.1, 4.2, 8.3 and 16.7 mM) for rat intestinal maltase in the presence of caffeic acid at (○) 0 μM, (□) 83.3 μM, (Δ) 166.7 μM, (●) 333.3 μM and (■) 666.7 μM.
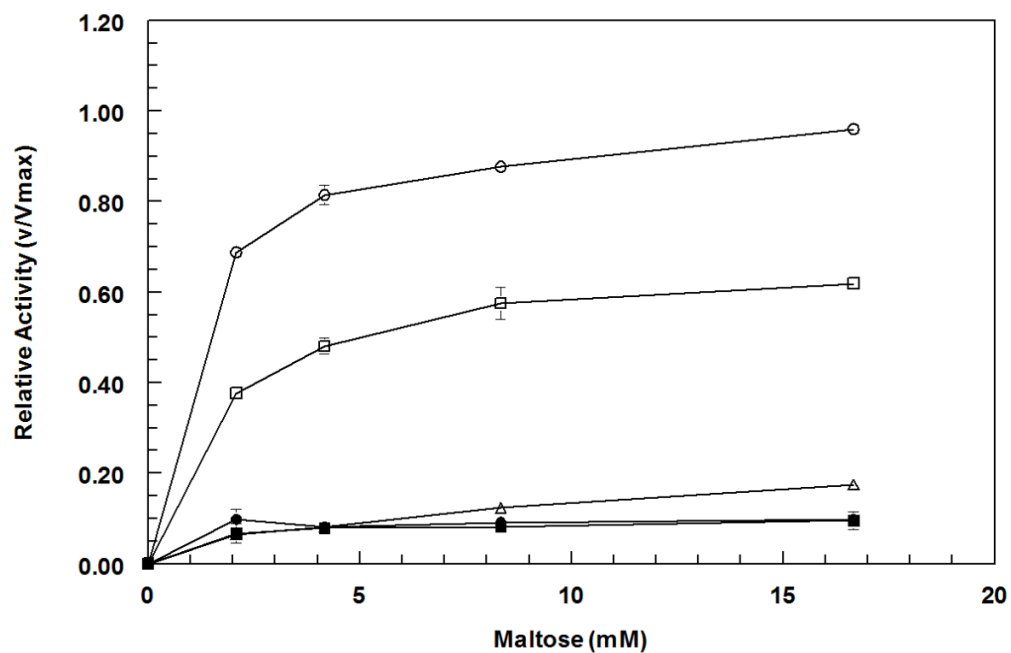
Figure 4.8 Relative activity (v/Vmax) change versus different maltose concentrations (2.1, 4.2, 8.3 and 16.7 mM) for rat intestinal maltase in the presence of gallic acid at (○) 0 µM, (□) 83.3 µM, (Δ) 166.7 µM, (●) 333.3 µM and (■) 666.7 µM.
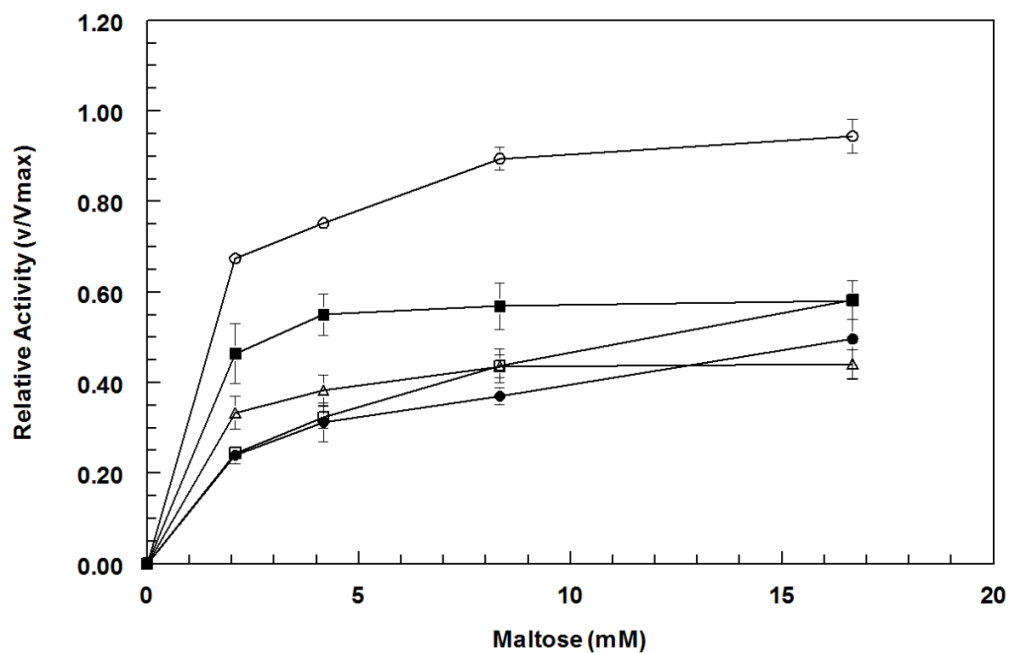
Figure 4.9 Relative activity (v/Vmax) change versus different maltose concentrations (2.1, 4.2, 8.3 and 16.7 mM) for rat intestinal maltase in the presence of (+)-catechin at (○) 0 μM, (□) 83.3 μM, (Δ) 166.7 μM, (●) 333.3 μM and (■) 666.7 μM.
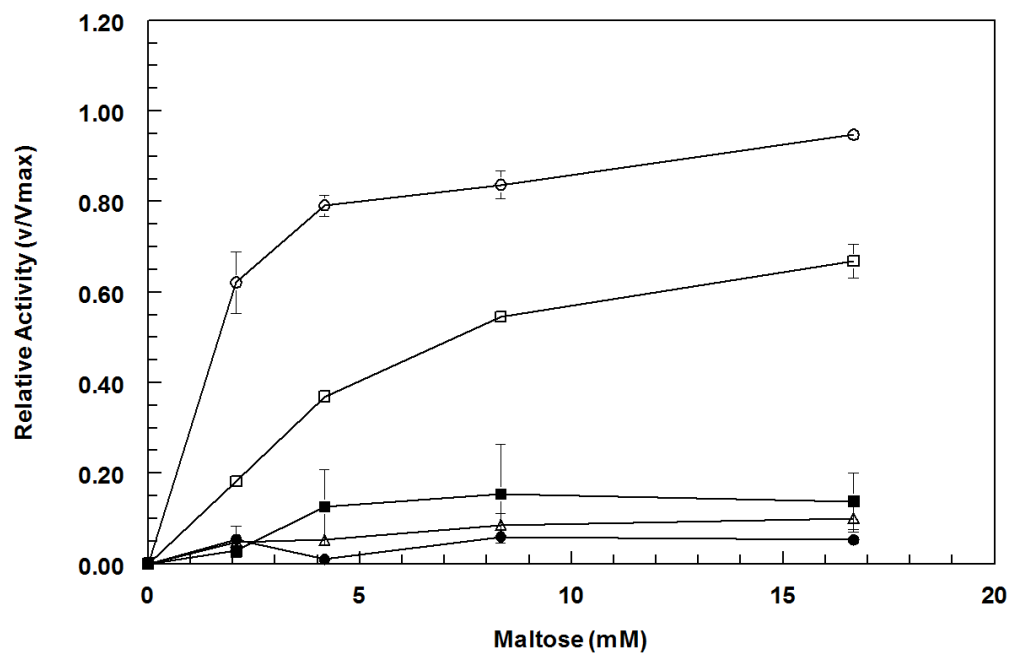
Figure 4.10 Relative activity (v/Vmax) change versus different maltose concentrations (2.1, 4.2, 8.3 and 16.7 mM) for rat intestinal maltase in the presence of chlorogenic acid at (○) 0 μM, (□) 83.3 μM, (Δ) 166.7 μM, (●) 333.3 μM and (■) 666.7 μM.
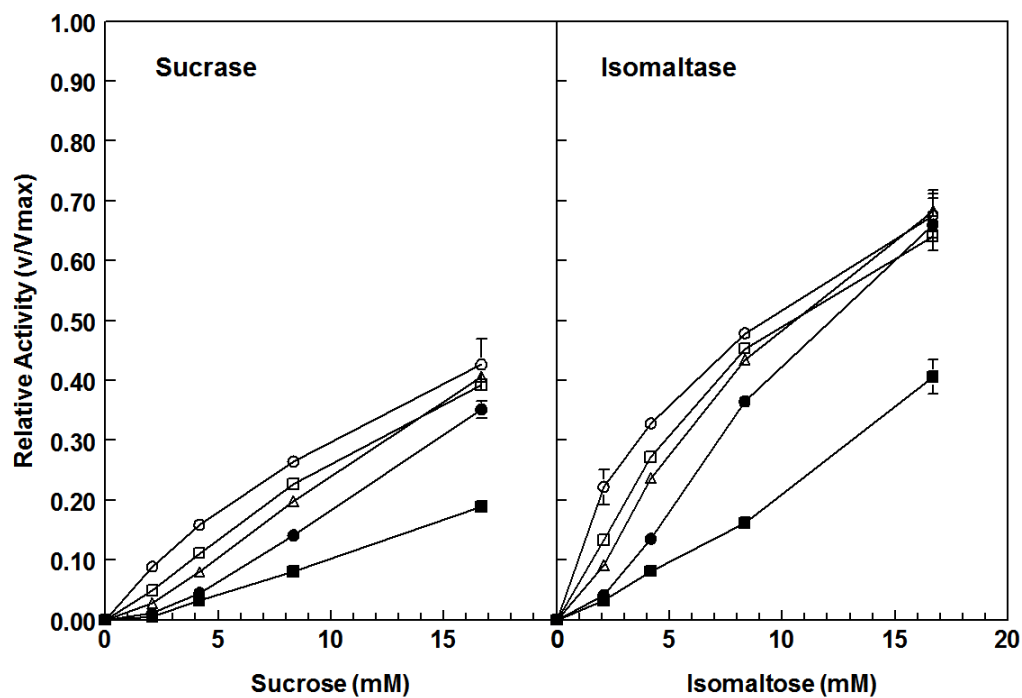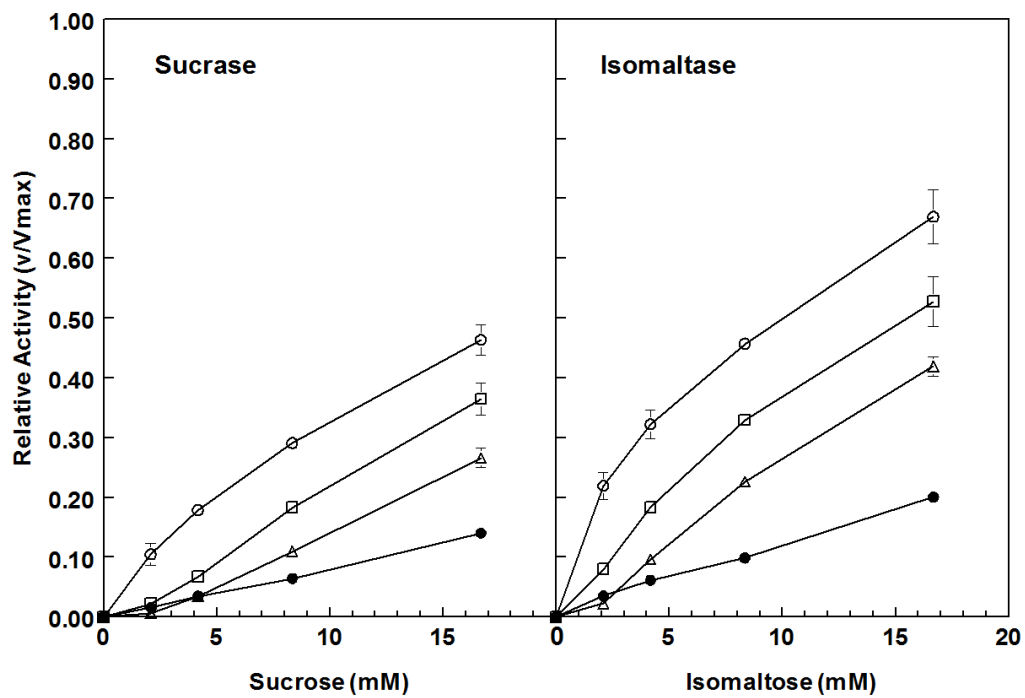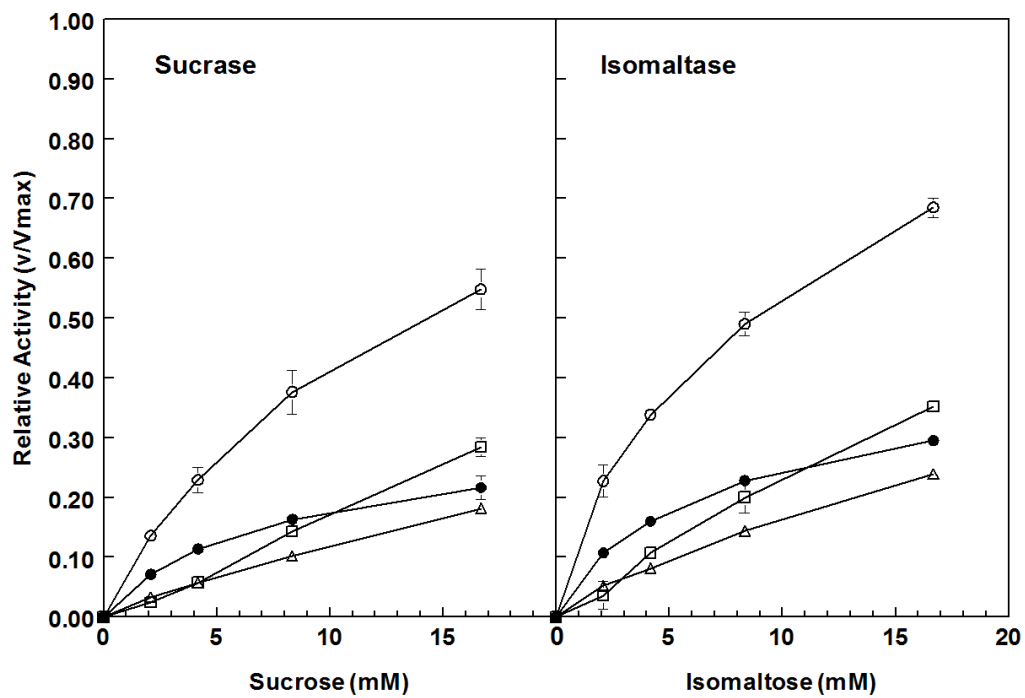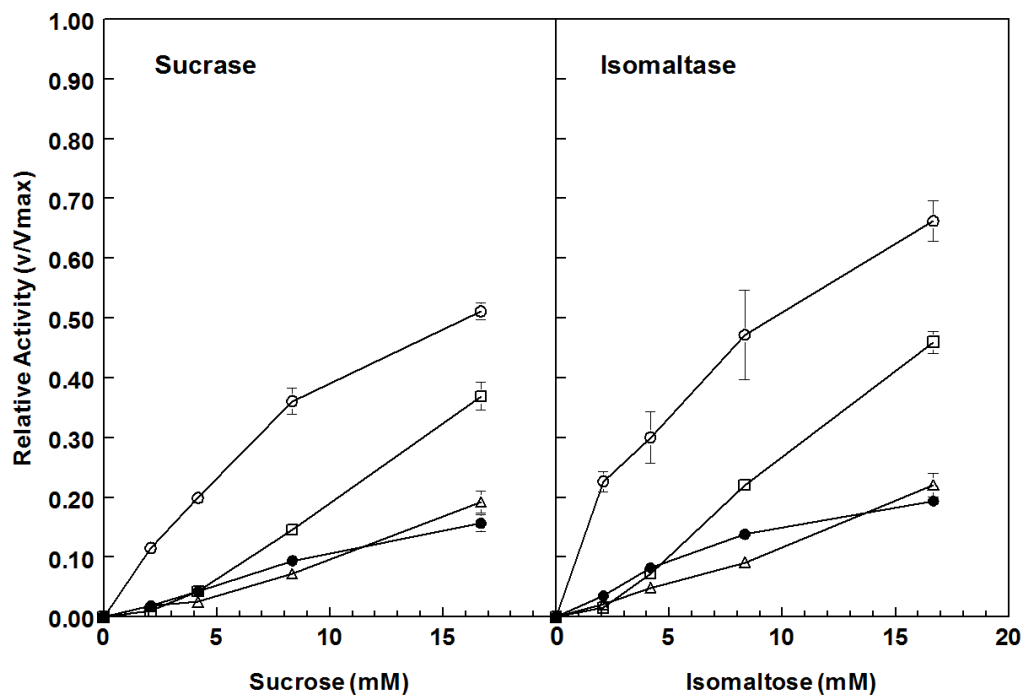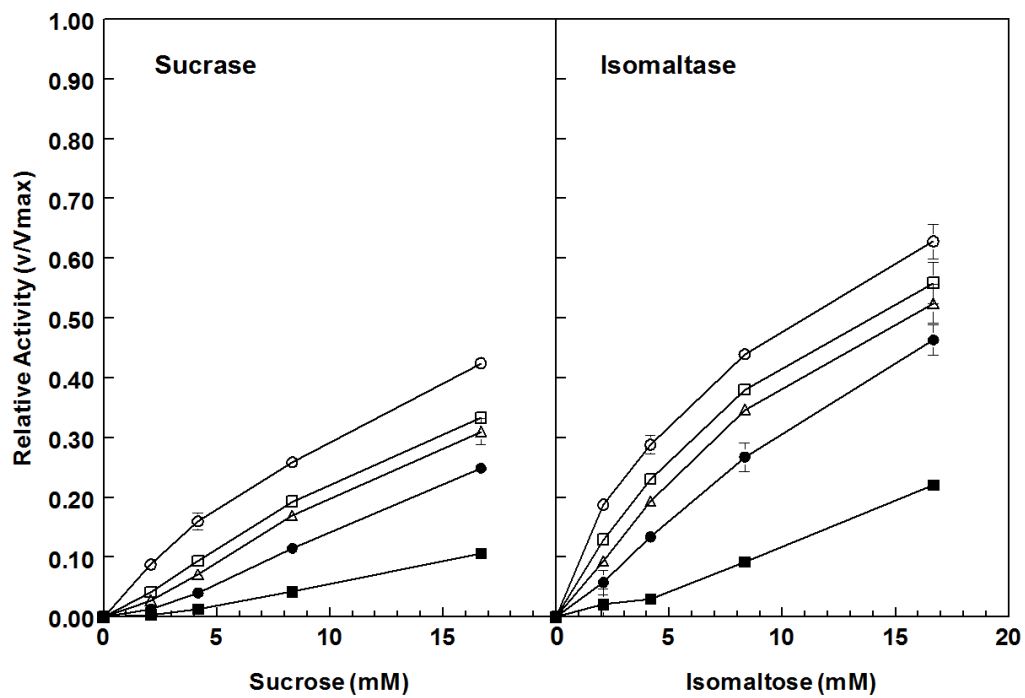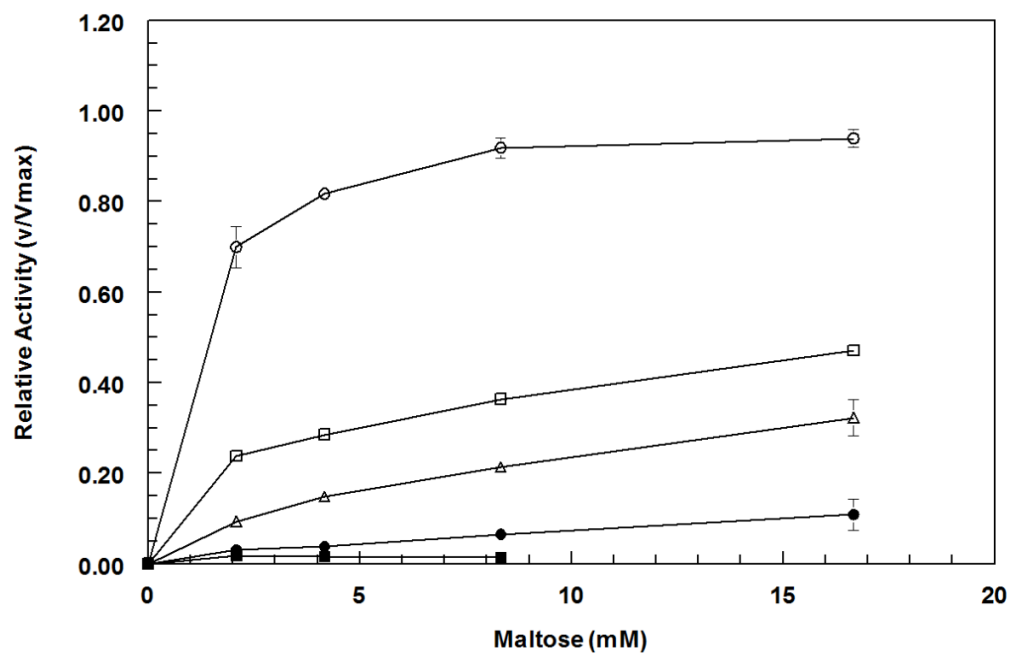
Figure 4.11 Relative activity (v/Vmax) change versus different maltose concentrations (2.1, 4.2, 8.3 and 16.7 mM) for rat intestinal maltase in the presence of EGCG at (○) 0 μM, (□) 10.4 μM, (Δ) 20.8 μM, (●) 41.7 μM and (■) 83.3 μM.

CHAPTER 5. DIETARY PHENOLIC COMPOUNDS INCREASE THE TRANSCRIPTION OF INTESTINAL MALTASE-GLUCOAMYLASE AND SUCRASE-ISOMALTASE

5.1   Abstract

Natural phenolics modify the expression of mRNA coding for proteins involved in nutritional, metabolic or immune processes. However, it was not known whether phenolic compounds influence the expression of α-glucosidase encoding genes for Maltase-Glucoamylase (MGAM) and Sucrase-Isomaltase (SI) located on the small intestinal brush border membrane. These enzymes are responsible for generating the free glucose from α-amylase digested starch. The aim of this study was to explore the effects of phenolic compounds on expression levels of MGAM mRNA and SI mRNA in mouse jejunal small intestinal explants. Small intestinal explants from 8 weeks old Balb/c mice were cultured 24 h in DMEM- 10% FBS media, in the presence or absence of gallic acid, caffeic acid and (+)-catechin at 0.1, 0.5 and 1 mM. mRNA coding for MGAM and SI was quantified by qRT-PCR analysis; the molecular size distribution of the protein products was determined by Western blot analysis; and total maltase activity was measured in treated tissues by the Tris glucose-oxidase peroxidase method. Among all compounds, caffeic acid at 0.1 mM was the strongest inducer for an increase in MGAM mRNA and

SI mRNA expression levels. In addition, different phenolic compounds affected the formation of different molecular weight forms of MGAM. However, no changes in the maltase activity of total jejunal small intestinal homogenates were induced by tested phenolic compounds. Results showed that phenolic compounds changed expression of MGAM and SI mRNA, and caused the generation of different molecular size forms of MGAM. However, these modifications had no effect on the overall maltase activity of the intestinal epithelium.

Keywords: gene expression, Maltase-Glucoamylase, phenolics, Sucrase-Isomaltase

5.2   Introduction

Starchy foods play a key role in supplying needed energy for the human body. In humans, salivary and pancreatic α-amylases hydrolyze starch into linear glucose oligomers and branched α-limit dextrins, but little free glucose (Quezada-Calvillo et al., 2008). These products are subsequently digested into the free glucose by the intestinal mucosal α-glucosidases, Maltase-Glucoamylase (MGAM) and Sucrase-Isomaltase (SI), which are expressed in intestinal enterocytes and trafficked to the small intestinal brush border membrane. Each of these enzymes is composed of two active subunits (C, Ct and N terminal, Nt). Both MGAM and SI have only α-1, 4-exoglucosidic activity (Quezada-Calvillo et al., 2008). While the glucoamylase subunit (Ct) of MGAM has higher activity on longer glucose oligomers, maltase (Nt) subunit activity favors the hydrolysis of shorter glucose oligomers (Quezada-Calvillo et al., 2008). Furthermore, it has been shown that glucoamylase subunit has higher α-glucosidic activity than the other subunits of MGAM and SI (Jones et al., 2011; Auricchio et al., 1965; Lin et al., 2012). The

sucrase (Ct) subunit of SI corresponds to the α-1, 2 glucosidic activity observed on sucrose, while the isomaltase subunit (Nt) is associated with the α-1, 6 activity; however, both of these enzymes also digest α-1, 4 linkages. In humans, SI, because of its relative higher abundance, accounts for 60-70 % of the total maltase activity. In contrast, despite substantially lower abundance of MGAM, the higher hydrolytic activity of Ct subunit of this enzyme allows it to contribute about 30% to the total intestinal maltase activity (Robayo et al., 2006).

To modulate the rate of glucose release in the intestinal brush border membrane, the inhibition of these enzymes has been studied (McDougall et al., 2005; Ishikawa et al., 2007; Eskandari et al., 2011). In Chapter 3, it was further shown that some phenolic compounds selectively inhibit the maltase activity of individual subunits of MGAM and SI. This suggests that the rate of glucose release from starchy products or sucrose can be modulated by phenolics, which could help to improve glycemic response particularly for some metabolic diseases such as diabetes. Importantly, there are some reports indicating that, in addition to chemical enzyme inhibition, diverse phenolics or their intermediate metabolites can modify the expression of genes related with energy metabolism, including glucose transporters or gluconeogenic enzymes (Cao et al., 2007; Sasaki et al., 2007; Abe et al., 2009, Qin et al., 2012). However, it is not known whether phenolics may also regulate the expression of MGAM and SI genes in the small intestine as an additional effect to the chemical inhibition of the activities of the corresponding enzymes. This would be of interest, because this additional effect could result in the modulation of the activities of MGAM and SI at a transcriptional level. Several studies have demonstrated that high-starch/low-fat diet induce the jejunal expression of MGAM and

SI mRNA and their intestinal enzyme activity, indicating that foods can deliver regulatory signals for these genes (Mochizuki et al., 2010; Honma et al., 2007).

Human MGAM is 1857 amino acids long with an estimated size of approximately 210 kDa (Nichols et al., 1998), and is encoded by a gene located on chromosome 7 (UCSC Genome Browser). In humans, the MGAM gene contains two tandem segments each coding for a whole C terminal subunit (http://genome.ucsc.edu). Similar gene arrangements have been observed in all other species in which MGAM gene has been sequenced. For instance, in mice, the whole MGAM gene expands a segment of close to 250 kbp of chromosome 6; this segment contains nucleotide sequences that encode for four different glucohydrolytic enzyme subunits all belonging to the GH31 family of glycohydrolases. The first region at the 5' extreme represents the N terminal of the mature protein which is anchored to the membrane; this is followed by three tandem regions each coding for a C terminal subunit (UCSC Genome Browser); these regions are then followed by a segment containing the potential stop codons which provide several alternative stop signals for the translation of the protein. This multi-subunit genomic arrangement requires alternative RNA splicing to generate the predominate form of the enzyme, which consists of two subunit structures that is commonly reported for intestinal MGAM. In humans, SI is encoded by a gene located on chromosome 3 (West et al., 1988) and generates a protein with estimated molecular size around 210 kDa (Moolenaar et al., 1995). After SI is produced, it is further glycosylated with N-linked and O-linked carbohydrate moieties (Moolenaar et al., 1995), and is then trafficked to the apical membrane of enterocytes. After its transport, free Ct-SI and membrane bound Nt-SI subunits are generated by extracellular pancreatic trypsin (Saphiro et al., 1991).

In this study, we explored the possible regulation of MGAM and SI gene expression, protein expression, and maltase activity induced by the selected phenolic compounds, gallic acid, caffeic acid and (+)-catechin. These phenolics have been shown to inhibit the activities of α-glucosidases. Therefore, there was an interest to investigate their potential effects on the expression of MGAM and SI genes.

5.3   Materials and Methods

5.3.1   Mouse small intestinal explant preparation

The experimental procedures used for the animals in this study were reviewed and authorized by the local Ethics Committee at University of San Luis Potosi (UASLP-FCQ-CEID2013009). All experiments were performed at least three times in an independent manner. In a typical experiment, four male Balb/c mice of eight-weeks of age were selected randomly, fasted overnight and then sacrificed. The entire jejunum was quickly removed, freed of all mesenteric tissues and its lumen thoroughly flushed with ice-cold 10 mM phosphate-buffered saline containing 2.5 mM of EDTA (PBS-EDTA) at pH 6.8. Explants were obtained from the cleaned intestinal segments by cutting them open longitudinally with round tip scissors and then cut into small square pieces of about 0.5 cm^2 (approximately 40 mg each).

5.3.2   Culture and harvesting of jejunal small intestinal explants

The small intestinal explants were placed in the wells of 48 well culture plates containing 1 ml of DMEM and 10% fetal bovine serum (FBS). Caffeic acid, gallic acid

and (+)-catechin, obtained from Sigma-Aldrich (St. Louis, MO, USA), were dissolved in deionized water and added to the individual wells containing the tissues fragments in enough volume to obtain final concentrations of 0.1, 0.5 and 1 mM. The control tissues were kept only in media solution without any added polyphenol. The plates were placed in an incubator with controlled concentration of CO_2 (5%) and humidity (95%) at 37 °C for 24 h. After incubation, the intestinal explants were harvested and each divided into two segments of approximated equal size. One segment was placed in ice-cold PBS-EDTA at 4 °C for the homogenization; the remaining part was immediately frozen in liquid nitrogen and then stored at -80 °C for the subsequent isolation of total RNA.

5.3.3   The homogenization of the jejunal small intestinal explants

Collected segments of intestinal explants were homogenized with a mortar and pestle in 40 µl of lysis buffer (10% sodium deoxycholic acid and 10% Nonidet P 40 solution in PBS-EDTA) with the additional 400 µl of PBS-EDTA (Quezada-Calvillo et al., 2007). The homogenated samples were used for measurement of protein concentration, quantitation of maltase activity, and analysis by SDS-polyacrylamide gel electrophoresis.

5.3.4   Determination of protein concentration in the jejunal small intestinal homogenates

Protein concentration in the jejunal small intestinal homogenates was measured using the Bio-Rad Protein Assay Kit (Hercules, CA, USA) using 10 µl of sample of homogenated tissue with 200 µl of dye reagent. The optical density was read at 595 nm

and the protein concentration in the samples was interpolated using a standard curve made with different concentrations of bovine serum albumin (BSA) from 0 to 500 µg/ ml.

5.3.5 Measurement of maltase activity in the jejunal small intestinal homogenates

Ten µl of diluted jejunal small intestinal homogenate were reacted with 10 µl of 50 mM maltose solution (25 mM final maltose concentration) for 1 h at 37 °C. After the incubation, the enzymatic reaction was stopped with the addition of 180 µl of Tris Glucose-Oxidase reagent (Dahlqvist, 1964; Quezada-Calvillo et al., 1993) and incubated for 45 min. The amount of glucose release was determined by reading the optical density at 450 nm on a Synergy HT microplate reader (BioTek; Winooski, VT, USA) with the aid of a standard curve built with glucose concentration from 0 to 400 µg/ml. Specific activity (U/mg protein) was calculated for each sample.

5.3.6 SDS-polyacrylamide gel electrophoresis and Western blotting

Twenty µl of samples containing exactly 10 µg of protein were loaded per lane of 4-15 % precasted TGX, Bio-Rad gradient polyacrylamide gels. Bio-Rad molecular weight markers (Precision Plus Protein Dual Color standards; 10-250 kD) were included in each gel. The resulting gels were transferred onto nitro-cellulose membranes (Bio-Rad) with a semi-dry transfer system and then blocked with 3% BSA overnight at 4 °C. The membranes were washed with 1X Tris–HCl buffered saline containing 0.1% Tween 20 (TTBS), and then allowed to react with rabbit anti-mouse MGAM and SI polyclonal antisera (B. Nichols, Baylor College of Medicine) (Ubelmann et al., 2013) diluted 1:2000 overnight at 4 °C. After extensive washing with TTBS, the membranes were reacted with

a second antibody, goat anti-rabbit IgG conjugated with biotin (SIGMA Immunochemicals), at 1:2500 of dilution for 1 h at room temperature. After being washed with TTBS, the membrane was reacted with conjugated avidine-alkaline phosphatase (SIGMA immunochemicals) at 1:2500 dilution. Later being washed again with TTBS, the bands were developed with AP conjugate substrate kit (Bio-Rad). The resulting blots were visualized, recorded, and analyzed densitometrically in a Chemi Doc system (Bio-Rad) with ImageLab® (Bio-Rad) software.

5.3.7   Total RNA isolation and cDNA synthesis

The acidified guanidine thiocyanate method was used to extract Total RNA with TRIzol reagent (Life Technologies; Carlsbad, CA, USA) (Chomczynski et al., 1987). The amount (ng/µl) and quality (ratio of optical density at 260/280 nm) of total RNA were determined on the microplate reader. Single-stranded cDNA's were generated from 1 µg of total RNA samples using high capacity cDNA reverse transcription kit (Applied Biosystems; Foster City, California, USA) according to its recommended protocol. The amount and quality of synthesized cDNAs were determined by measuring the absorbance of the solution at 260 and 280 nm.

5.3.8   Quantitative Real Time PCR (qRT-PCR)

The mRNA expression levels of MGAM and SI were quantified by the Bio-Rad qRT-PCR system. Real-time PCR reactions were performed in a total volume of 20 µl containing 100 ng of cDNA, SsoFast EvaGreen supermix (Bio-Rad), and 500 nM of each forward and reverse specific primer. The sequences of oligonucleotide primers were as

follows: MGAM, 5'-GCC AAG GTT ACA AGG ACC AG-3' (forward) and 5'-TAA GGC AGC AAA GTG TAG CG-3' (reverse) which yielded an expected product of 103 bp; SI, 5'-CGT TTC CGG TTC AAG CTC ACA-3' (forward) and 5'-CCT GAT GAC TTT GAT GCT GAA CG-3' (reverse) with a expected product of 147 bp. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping or reference gene expression using the primers, 5'-TCT GGA AAG CTG TGG CGT G-3' (forward) and 5'-CCA GTG AGC TTC CCG TTC AG-3' (reverse) with a expected product of 116 bp. RT-PCR conditions were an initial activation period at 95 °C for 30 s; 40 amplification cycles at 95 °C for 5 s, and then either 56.3, 50 or 50.7 °C for MGAM, SI, and GAPDH, respectively, for 5 s and the range of 65 to 95 °C for 5 s. Relative amount of mRNA was normalized to GAPDH. Relative mRNA expression was compared with that of control tissues not exposed to phenolic compounds and the results expressed as ΔΔCq.

5.4   Results

5.4.1   The effects of phenolic compounds on the jejunal expression of the MGAM gene in mice

We investigated the effects of the exposure of mice jejunal small intestinal explants to phenolic compounds (gallic acid, caffeic acid and (+)-catechin) in different concentrations (0.1, 0.5 and 1 mM) on the expression of MGAM mRNAere exposed to . Compounds showed different potencies for the induction of expression of jejunal MGAM mRNA. As shown in Figure 5.1, gallic acid, caffeic acid and (+)-catechin treatments

caused up-regulation of MGAM genes in the explants; many were significant in comparison with the control without any polyphenol treatment ($p<0.05$). Exposure to gallic acid at 0.1 mM only, but not 0.5 or 1 mM, resulted in a significant 0.49-fold increase in the jejunal MGAM mRNA expression as compared to the control (Figure 5.1). For the caffeic acid treatment, jejunal expression of MGAM mRNA was 0.82-fold, 0.63-fold and 0.63-fold higher at 0.1, 0.5 and 1 mM, respectively, than in those had no polyphenol treatment ($p<0.05$, Figure 5.1). (+)-Catechin at the concentration of 0.1 mM and 0.5 mM only, but not 1 mM, increased jejunal expression of MGAM mRNA by 0.55-fold and 0.53-fold ($p<0.05$). The results showed that caffeic acid at 0.1 mM was the strongest inducer of the expression of MGAM mRNA among all tested phenolic compounds at different concentrations. In addition, the best induction in jejunal expression of MGAM mRNA caused by phenolic compounds was attained with the lowest concentration of 0.1 mM. Using the lowest concentration of 0.1 mM to compare the potency of the tested phenolic compounds for induction of jejunal expression of MGAM mRNA, they were ranked in order as: caffeic acid > (+)-catechin > gallic acid.

5.4.2   The effects of phenolic compounds on jejunal expression of SI mRNA in mice

We also analyzed the induction of SI mRNA expression in mouse jejunal small intestinal explants caused by the same exposure to phenolic compounds. The jejunal expression of SI mRNA was increased 0.54-fold by gallic acid only at 0.5 mM as compared to the control without phenolic treatment ($p<0.05$). The level of induction of jejunal expression of SI mRNA by caffeic acid was comparable to the one seen in the jejunal expression of MGAM mRNA, and again the lowest concentration level had the

highest expression effect [0.1, 0.5 and 1 mM of caffeic acid provided 0.88-fold, 0.51-fold and 0.53-fold higher jejunal expression of SI mRNA than the control without any polyphenol treatment ($p < 0.05$, Figure 5.2)]. Suprisingly, (+)-catechin did not show significant change in the expression of SI mRNA at all tested concentrations (Figure 5.2). Overall, caffeic acid was the strongest inducer in the increment of jejunal expression of MGAM and SI mRNA.

5.4.3 Western blot analysis of MGAM and SI in the presence of phenolic compounds

To examine the possible modifications in the protein synthesis or processing of MGAM or SI caused by gallic acid, caffeic acid or (+)-catechin, homogenates of mice jejunal small intestinal explants were analyzed by Western blotting (WB). Since comparable results were obtained from WBs analysis performed in samples of four independent experiments, we present Figure 5.3 as a representative example of them. MGAM in control tissue not treated with phenolic compounds showed the characteristic bands pattern observed for mouse MGAM in high resolution SDS-PAGE which consists of three groups of bands: the first group contained three closely spaced bands with molecular size in the range of 390 to 410 kDa, which are consistent with molecular forms containing the four MGAM subunits (Figure 5.4); the middle group includes two bands with molecular size in the range of 240 to 260 kDa, which correspond to the generally reported molecular forms of two MGAM subunits (Figure 5.4); and the third group, also formed by two bands with molecular size of 110 to 130 kDa, which are consistent with molecular forms containing each of one single subunit (Figure 5.4) (Flanagan et al., 1978). We analyzed the changes in different forms of MGAM by the relative proportion

of each of these groups in the polyphenol-treated samples as compared with the control untreated sample (Figure 5.3). As seen in the lane of control sample (Ctrl) of Figure 5.3, the low molecular weight (MW) one subunit form was in the highest proportion, followed by the four subunits form, and then two subunits form. WB analysis demonstrated that gallic acid at concentration of 0.1, 0.5 and 1 mM increased the proportion of the four subunits form as well two subunits form compared to the control; and the proportion of the one subunit form decreased (Figure 5.3 and 5. 5). Among the different doses of gallic acid, the lowest concentration of gallic acid resulted in the highest increase in the four and two subunit forms, and also the highest decrease in the one subunit form (Figure 5.3 and 5.5).

Changing in banding patterns of MGAM in the presence of the different concentrations of gallic acid, caffeic acid, and (+)-catechin could also be related to specific band changes representing each different subunit form of MGAM. For instance, in case of gallic acid, the increase in the proportion of high molecular weight four subunits form were mainly caused by the top band in triplicate band groups of the four subunits form whereas the increase in the proportion of two subunits form of MGAM were mainly caused by lower band in duplicate band group of the two subunits form, respectively (Figure 5.3). However, the decrease in the proportion of the one subunit form of MGAM was caused by decrease of the individual bands at approximately equal levels.

In case of caffeic acid, 0.1 and 0.5 mM did not change the proportion of the four subunits form, but increased the proportion of the two subunits form and decreased the one subunit form as compared to the control (Figure 5.3 and 5.5). Caffeic acid at 1 mM

decreased the proportion of four subunits form, and increased two subunits form and did not change the proportion of one subunit form. Caffeic acid at 0.1 and 0.5 mM concentration shifted the proportion of the two subunit form of MGAM higher compared to the caffeic acid at 1 mM (Figure 5.3), and this increase was caused mainly by an increase in the lower individual band in the duplicate band group representing the two subunit form of MGAM. (+)-Catechin at 0.1, 0.5, and 1 mM concentration increased the proportion of the four subunit and two subunit forms and decreased the one subunit form that was caused mainly due to its individual top band of four subunits form, lower band of two subunits form and each of individual bands of one subunit form (Figure 5.3 and 5.5).

Overall, results indicated that the relative proportion of the formation of different subunit forms or different molecular size forms of MGAM was altered from the control, because of the exposure of tested phenolic compounds. Also, we found that all tested phenolic compounds increased the proportion of the two subunit form of MGAM (the 240-260 kDa form), as compared to the control. This is the considered form that is active at the enterocyte apical membrane surface to digest α-amylase degradation products of starch (α-limit dextrins and linear maltooligosaccharides). Even though there were changes in the proportion of different molecular size forms of MGAM, the proportion of the one subunit form of MGAM was still dominant in all polyphenol treatments, as was seen in the control. Differences within each band group, particularly in the two subunits and one subunit form, due to different polyphenol treatments may be important related to the existence of different glycosylated forms of MGAM.

As seen in Figure 5.6, there were two sets of bands corresponding to 250-260 and 120-130 kDa in WB of SI. While our results showed that there were changes in the proportion of different molecular size forms of MGAM with different phenolic compounds, they did not appear to cause any significant changes in the proportion of the different molecular size forms of SI (Figure 5.6).

5.4.4 Maltase activity in the jejunal small intestinal homogenates

Despite the finding that MGAM and SI mRNA expression was significantly upregulated by gallic acid, caffeic acid, and (+)-catechin, there was no significant change in maltase activity of the α-glucosidases. Maltase activity was measured in homogenates obtained from the same mice jejunal small intestine explants generated in four independent experiments (Figure 5.7).

5.5 Discussion and Conclusions

In the present study, we examined the effect of phenolic compounds, gallic acid, caffeic acid, and (+)-catechin at three different concentration levels (0.1, 0.5 and 1 mM) on jejunal MGAM and SI mRNA expression, on the different molecular forms of MGAM and SI found in the small intestine, and on overall small intestinal maltase activity. This is the first study demonstrating that jejunal MGAM and SI mRNA expression can be upregulated by minor dietary components such as natural phenolic compounds, instead of macronutrients as was shown before for starch-rich diets (Mochizuki et al., 2010; Honma et al., 2007). In a related study on glucose uptake, GLUT2 and SGLT1 mRNA expression

in Caco-2 cells was found significantly reduced with the exposure of berry extract rich in anthocyanins; although they did not observe a significant change in total glucose uptake (Alzaid et al., 2013).

We found that caffeic acid of 0.1 mM resulted in the highest up-regulation of the jejunal MGAM mRNA expression. Jung et al. (2006) showed that caffeic acid up-regulated the expression of glucokinase mRNA and down-regulated glucose-6-phosphatase and phosphoenolpyruvate carboxykinase mRNA expressions, also with a decrease in the glucose transporter 2 expression in the liver. (+)-Catechin and gallic acid also showed up-regulation of MGAM mRNA by exposure at the dose of 0.1 mM. In this study, the lowest concentration of all tested compounds was the best inducer. It might be that the higher dosages of 0.5 and 1 mM may have caused an overload of the signal transduction mechanisms involved in the induction of the expression of jejunal MGAM and SI mRNA. All tested compounds caused similar up-regulating levels for jejunal expression of MGAM and SI mRNA. The up-regulating effect of phenolic compounds on the jejunal expression of these proteins is promising for investigating the poorly characterized intracellular signaling mechanism controlling the expression of brush border membrane proteins, in particular MGAM. There is little information available in the published literature about the transcriptional regulation of the MGAM gene.

While up-regulating the jejunal expression of MGAM and SI mRNA by tested phenolic compounds, they also caused changes in the relative proportion of the different molecular forms of MGAM (called here four subunits, two subunits, and one subunit form of MGAM), as compared to the control. This modification in the distribution patterns of molecular forms strongly suggests that the synthetic pathways of these

enzymes experienced alterations. There could be a few different explanations for this occurrence, and as well for the existence of these three different MGAM forms. The nature of the MGAM gene architecture implies that the formation of a putative four subunit protein with MW of about 450 kDa requires the simple splicing of introns from the primary transcript of the gene; while, in contrast, the generation of a two subunits protein would require alternative splicing of equivalent exons to generate a single segment coding for the Ct subunit in the mature mRNA (Figure 5.4). Alternatively, a four subunit protein precursor could be subjected to proteolytic posttranslational processing rendering membrane bound and soluble proteins forms each containing two subunits and MW of 250 kDa (Figure 4). Additional proteolytic posttranslational processing could also account for the production of the single subunit forms (110-130 kDa) representing individual N-terminal or C-terminals subunits (Figure 5.4). The higher proportion of the two subunit form of MGAM corresponding to 240-260 kDa observed in the presence of all phenolic compounds, this indicates that the enterocyte adjusted to generate more of this form which as a result of either alternative splicing or posttranslational processing (Figure 5.4). This may be related to the generally accepted view that the two subunit form, consisting of one Nt-MGAM and one Ct-MGAM, is the active form found at the apical surface of the enterocyte; and, in the presence of the polyphenol inhibitor, the reason of this adjustment may be because of the need to maintain the required level of enzymatic activity in the small intestinal brush border membrane. This points to a need to investigate the underlying mechanism of synthesis, posttranslational processing and transport of MGAM causing alterations in the proportions of different molecular size forms of the enzyme.

Despite the observed up-regulation of jejunal expression of MGAM mRNA and changes in molecular forms, these changes did not generate significant differences in the overall maltase activity. This may be related to a more defined requirement for higher level of the active two subunit MGAM form, but may also be related to a change in the cellular regulation and induction of changes in the rate of MGAM synthesis and posttranslational processing of the molecules. It has been proposed the existence of alternative spliceforms of MGAM with different catalytic properties, which could mask the increment of MGAM molecules without an increment on the overall maltase activity (Jones, 2011), and that would be formed to be active in the presence of polyphenolic inhibitors.

Unlike the MGAM protein, the synthesis, processing, and intracellular transport of SI protein has been widely studied and reported. SI is produced as a single polypeptide with the glycosylation of N-linked and O-linked carbohydrates (Moolenaar et al., 1995). This glycosylation is needed to transport SI to the apical membrane of enterocytes where it is exposed to the extracellular pancreatic proteases to produce free Ct-SI and membrane-bound Nt-SI subunits (Saphiro et al., 1991). However, these subunits still interact with each other non-covalently to form an intact complex. MGAM is attached to the apical membrane of enterocytes by its N terminal end. Although, it has not been documented that MGAM undergoes proteolytic processing as similar to SI, there are likely similar processing events. In the case of SI, there is no evidence yet of the existence of spliceforms at a genomic or structural level. Despite of the observed changes in SI mRNA expression, the distribution of 250-260 and 120-130 kDa forms of SI did not change in the presence of phenolic compounds.

In conclusion, the tested phenolic compounds up-regulated the expression of jejunal MGAM and SI expression, changed the proportion of different molecular forms of MGAM, but not SI, while overall maltase did not change. This study shows that phenolics, in addition to macronutrients such as starch, can regulate the jejunal expression of MGAM and SI. While it may be speculated that such alterations to the mucosal α-glucosidases are ultimately for the purpose of more efficiently digesting starch in the presence of polyphenolic inhibitors, further study is required to understand the underlying inhibitor effect on synthesis, posttranslational processing, and transport of MGAM.

5.6 <u>References</u>

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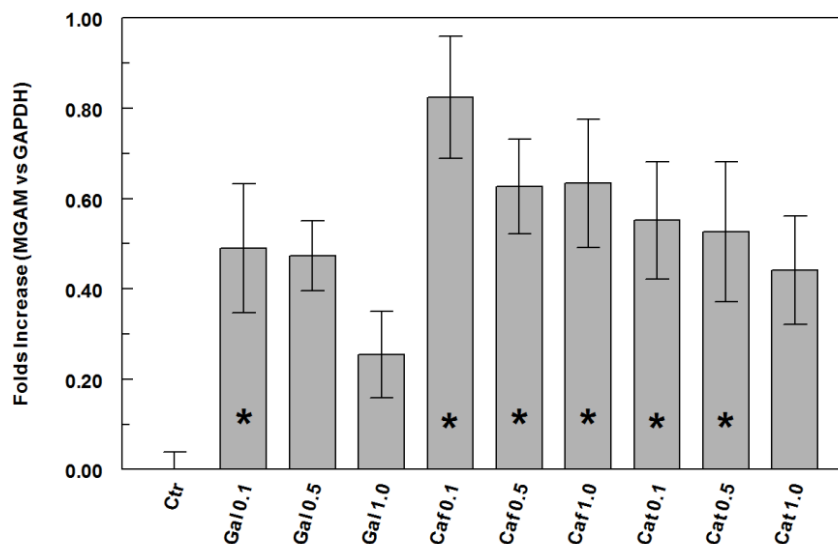
Figure 5.1 Quantitative expression of jejunal MGAM mRNA in the small intestinal tissues exposed to no polyhenolic compound (Ctr), gallic acid (Gal), caffeic acid (Caf) and (+)-catechin (Cat) at 0.1, 0.5 and 1 mM. As compared to the control, the treatments showing a significant increase ($p<0.05$) in jejunal expression of MGAM mRNA are shown with "*" inside the bar.
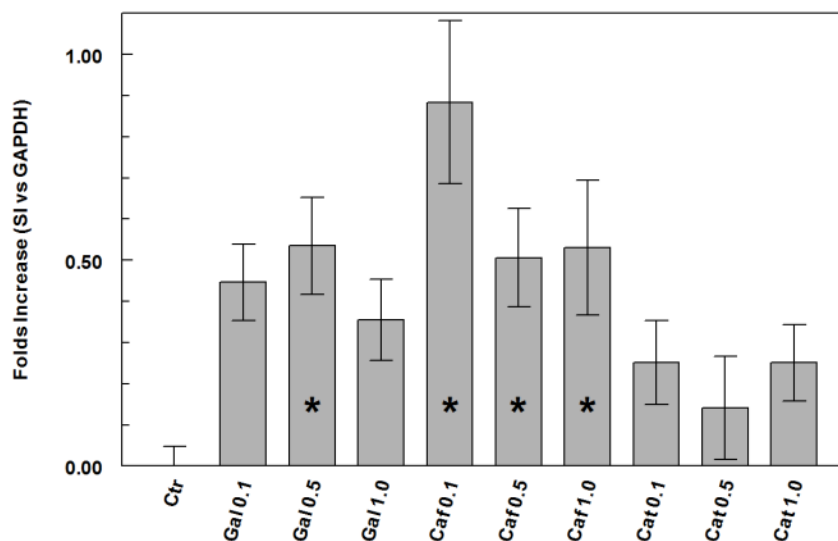
Figure 5.2 Quantitative expression of jejunal SI mRNA expression in the small intestinal tissues exposed to no polyhenolic compound (Ctr), gallic acid (Gal), caffeic acid (Caf) and (+)-catechin (Cat) at 0.1, 0.5 and 1 mM. As compared to the control, the treatments showing a significant increase (p<0.05) in jejunal expression of MGAM mRNA are shown with "*" inside the bar.
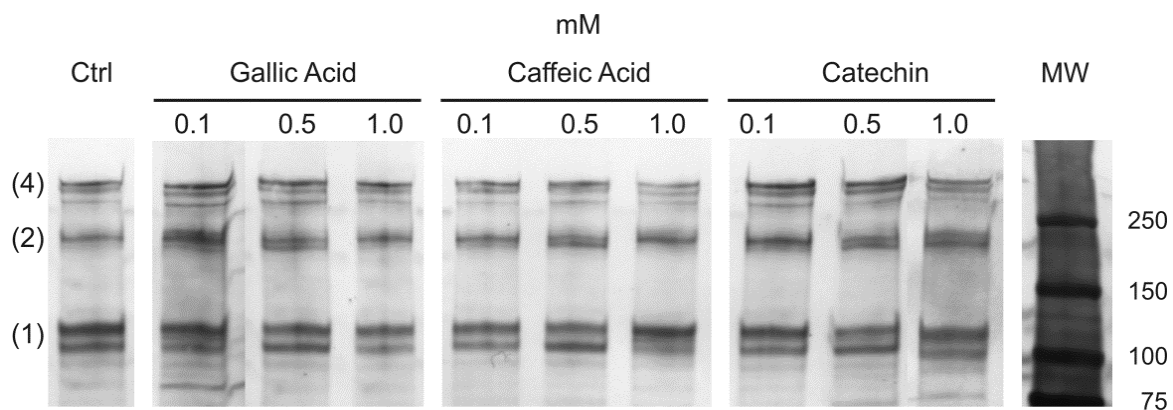
Figure 5.3 Western blot showing the effect of gallic acid, caffeic acid, and (+)-catechin on mouse MGAM protein. The lanes from the left to the right is as followed: the control (Ctrl), gallic acid (0.1, 0.5 and 1 mM), caffeic acid (0.1, 0.5 and 1 mM), (+)-catechin (0.1, 0.5 and 1 mM) and molecular weight marker (MW in kDa). The four subunits, two subunits and one subunit are represented with (4), (2) and (1) on the left side of Ctrl.
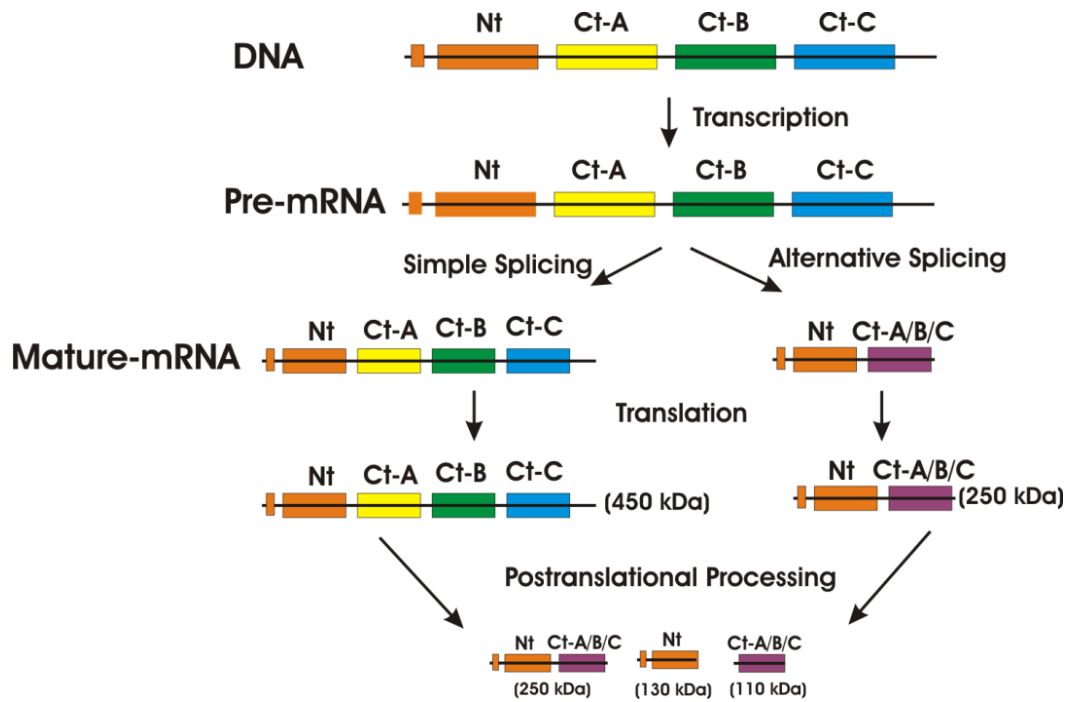
Figure 5.4 Hypothetic expression pathways of MGAM. Nt and Ct correspond to the N and C terminal of MGAM protein. A, B and C represents different C terminal segments.
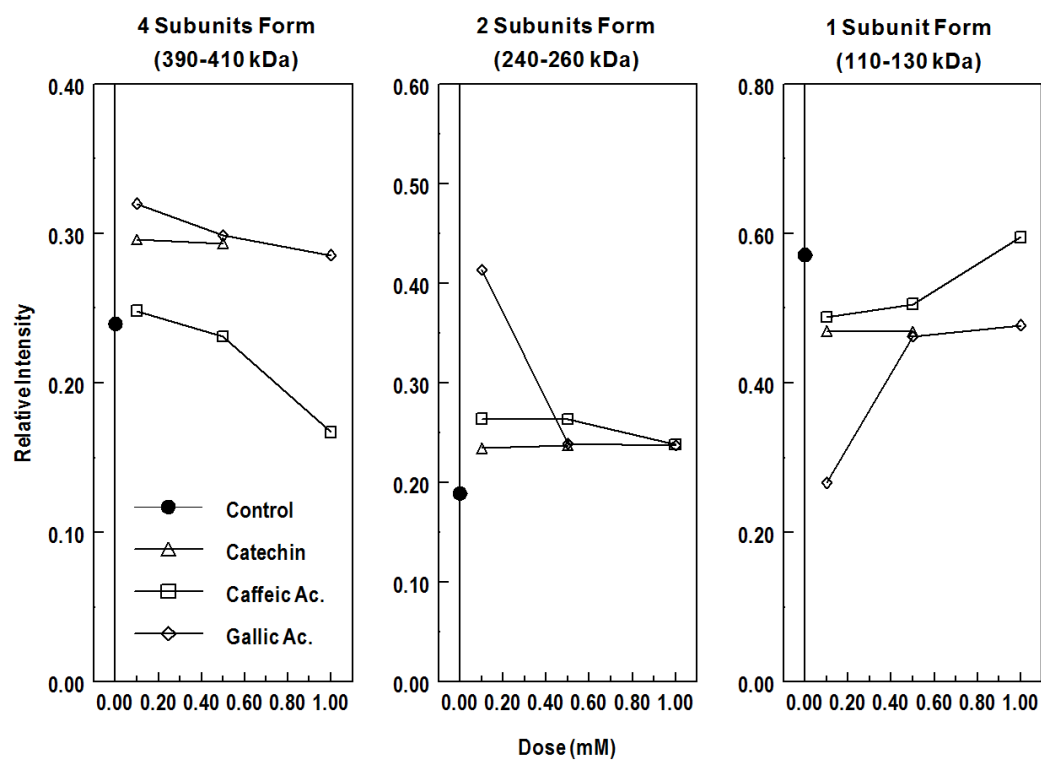
Figure 5.5 The change in the proportion of relative intensity corresponding to the different molecular size forms of MGAM (4 subunits from, 390-410 kDa; 2 subunits form, 240-260 kDa; and 1 subunit form,110-130 kDa) at different concentrations (0.1, 0.5 and 1 mM) of gallic acid, caffeic acid and (+)-catechin as compared to the control.
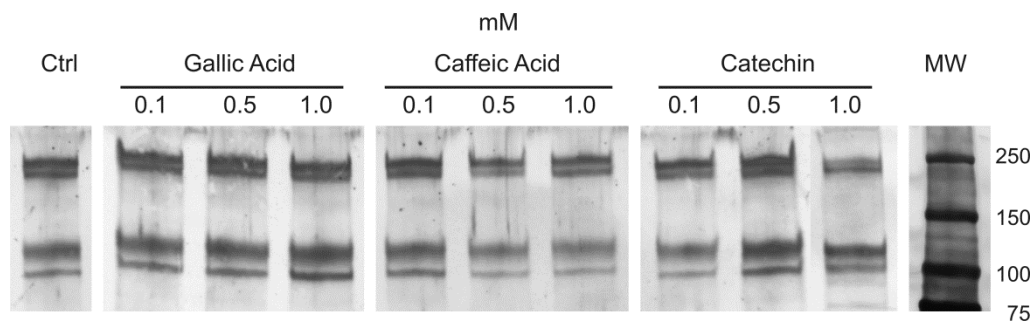
Figure 5.6 The western blot showing the effect of gallic acid, caffeic acid, and (+)-catechin on mouse SI protein. The lanes from the left to the right is as followed: the control (Ctrl), gallic acid (0.1, 0.5 and 1 mM), caffeic acid (0.1, 0.5 and 1 mM), (+)-catechin (0.1, 0.5 and 1 mM) and molecular weight marker (MW in kDa).
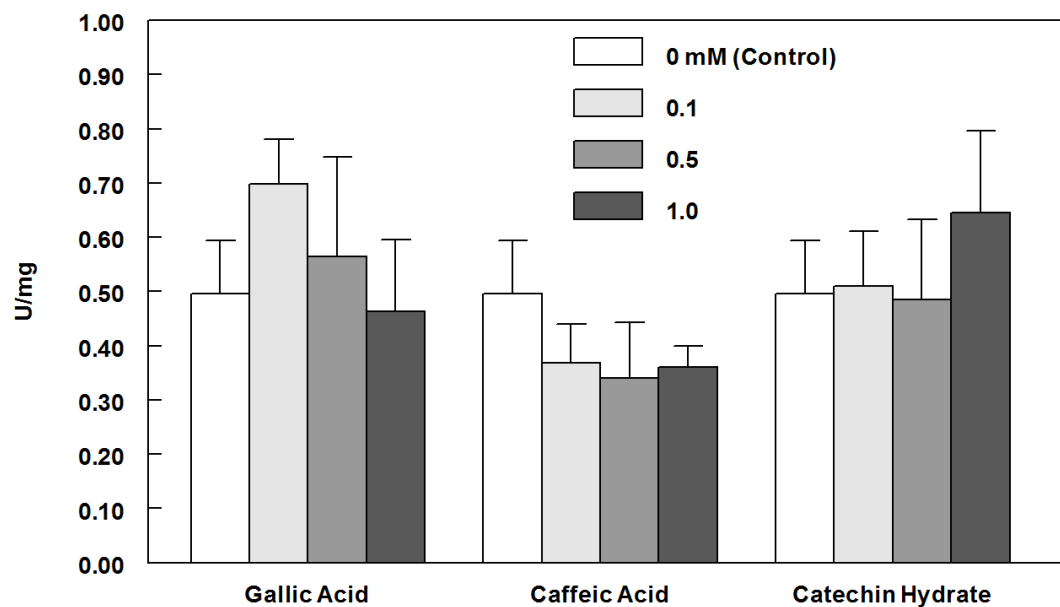
Figure 5.7 The effects of treatments [no polyphenol (0 mM), gallic acid (0.1, 0.5 and 1 mM), caffeic acid (0.1, 0.5 and 1 mM) and (+)-catechin (0.1, 0.5 and 1 mM)] on the maltase activity of mouse MGAM and SI.

CHAPTER 6. DISTINCT INHIBITORY POTENCIES OF DIETARY PHENOLIC COMPOUNDS FOR THE MUCOSAL ALPHA-GLUCOSIDASES IN THE HUMAN SMALL INTESTINE

6.1 Abstract

The enzymatic activity of intestinal α-glucosidases can be inhibited by dietary phenolic compounds. This study aimed at defining the inhibitory potencies of selected phenolic compounds on maltase activity of human small intestinal lysate, human immunoprecipitated Maltase-Glucoamylase (MGAM-IP) and Sucrase-Isomaltase (SI-IP), as well as sucrase activity of SI-IP. The inhibition mechanism and inhibition constant of tested phenolic compounds (caffeic acid, gallic acid, (+)-catechin, chlorogenic acid and (-)-epigallocatechin gallate (EGCG)) were also determined for the above mentioned activities. Glucose release was determined by the Tris glucose-oxidase peroxidase method. Results showed that the sucrase activity of SI-IP was comparatively highly inhibited by chlorogenic acid among all tested compounds. EGCG inhibited the maltase activity of MGAM-IP. These results supported our previous results showing phenolic compounds can selectively inhibit the individual subunits of MGAM and SI. Therefore, chlorogenic and EGCG can be considered as promising compounds to modulate glucose release from sucrose and starch and starch-derived products in the human small intestine. Keywords: inhibition, human, maltase, phenolics, sucrase

6.2 Introduction

Of the glycemic carbohydrates that humans use for energy, starch may be one of the main components found in most diets. Starch digestion has become more important as a target for researchers since its control may help in the management of patients with some metabolic diseases such as diabetics and cardiovascular disease associated with obesity. The starch-degrading enzymes are salivary and pancreatic α-amylases, generating linear glucose oligomers, branched α-limit dextrins and very little free glucose (Quezada-Calvillo et al., 2008), and the α-glucosidases, Maltase-Glucoamlyase (MGAM) and Sucrose-Isomaltase (SI) convert α-amylase degraded products into free glucose. MGAM and SI are located on the small intestinal brush border membrane (Quezada-Calvillo et al., 2007) and belong to the GH31 family of glucohydrolases. Even though MGAM and SI show 59 % similarity in their amino acid sequence, and have high degree of homology within their catalytic subunits (40-60 %) (Jones et al., 2011), individual subunits of MGAM and SI can be differentiated by their unique specific activities. For instance, while sucrase and isomaltase subunits of SI cleave α-1,2 and α-1,6 linkages of sucrose and isomaltose, respectively, MGAM does not have sucrase activity and shows very little isomaltase activity. However, all four subunits of MGAM and SI share the α-1,4 glucosidic activity.

The inhibition of the activities of MGAM and SI is one of the approaches to modulate the rate of starch and sucrose digestion and accompanying glucose release in the small intestine. There have been various in vitro and in vivo studies showing the influence of phenolics for reducing the rate of starch or sucrose digestion. In vitro studies

using rat intestinal acetone powder have been used to investigate the inhibitory effect of phenolics on the activity of α-glucosidases (Gupta et al., 2007; Honda et al., 1993; Xu et al., 2013; Koh et al., 2010; Adisakwattana et al., 2009; Ishikawa et al., 2007). In human studies, consumption of foods rich in phenolics was reported to result in the modulation of the postprandial glycemic response (Torronen et al., 2010; Hlebowicz et al., 2007). So far, inhibition studies have used mostly α-glucosidases obtained from yeast and the rat small intestine to test inhibitory effects of dietary phenolic compounds, or extracts of plants and fruits rich in phenolics, to control the rate of glucose release. Here, our main objective was to investigate for the first time the effect of phenolics on α-glucosidases of human small intestine tissue to modulate glucose release. The inhibition mechanism and inhibitory potencies were studied on selected compounds (gallic acid, caffeic acid, (+)-catechin, chlorogenic acid, epigallocatechin gallate) on the maltase activity of human small intestinal lysate, MGAM-IP, SI-IP, and sucrase activity of SI-IP.

6.3   Materials and Methods

6.3.1   Materials

D-(+)-maltose monohydrate and sucrose were purchased from Sigma-Aldrich. Gallic acid, caffeic acid, chlorogenic acid, (+)-catechin and (-)-epigallocatechin gallate (EGCG) were also obtained from Sigma-Aldrich.

6.3.2 Human small intestinal mucosal collection, homogenization and lysis

Human small intestine from an organ donor was obtained in agreement with H-1614 approval from the Baylor College of Medicine Institutional Review Board (Quezada-Calvillo et al., 2007). The enterocytes were scraped from the jejunal surface with a glass slide and concentrated by centrifugation. The obtained pellet was frozen at -70 °C. An aliquot of this pellet was used for enzyme inhibition analysis. The homogenates were prepared in phosphate-buffered saline (PBS) solution and lysed with 10% sodium deoxycholic acid and 10% Nonidet P 40 solution.

6.3.3 Immunoprecipitation of MGAM and SI

The human small intestinal mucosa lysate was centrifuged for 30 min at 100,000 g. This lysate was pre-cleared with unbound ProtA beads. Two aliquots of raw ProtA beads (GE Healthcare, Little Chalfont, United Kingdom) were first bound with antibodies. The first aliquot was bound with the anti-human MGAM monoclonal antibodies LAMA 127, LAMA 77 and LAMA 207 previously used for the immunoprecipitation of MGAM [14]; and the second aliquot was bound with the anti-human SI monoclonal antibodies HSI 3/42/1/2 and HSI 3/56/4/1 for the immnunoprecipitation of SI (Quezada-Calvillo et al., 2007; Quaroni et al., 1985). These aliquots of Prot A beads with bound antibodies were washed three times and finally resuspended at 50% v/v with binding buffer containing 1% extraction buffer (1% sodium deoxycholic acid and 10% Nonidet P 40 solution in PBS-EDTA). The pre-cleared lysate was added to the beads attached with the specific antibodies and the mixture was incubated for 2 h at 4 °C on a rotating mixer.

Finally, the recovered pellet was resuspended in PBS-EDTA and used for the inhibition assays.

6.3.4 Determination of protein concentration in the jejunal small intestinal homogenates

Protein concentration of the human small intestinal lysate was determined using the Bio-Rad Protein Assay Kit (Hercules, CA, USA). Ten μl of lysate sample was incubated with 200 μl of dye reagent. After incubation, the optical density was read using a Synergy microplate reader (Biotek; Winooski, VT, USA). The protein concentration in the lysate sample was calculated using a standard curve obtained with a concentration range (0-500 μg/ml) of bovine serum albumin.

6.3.5 Inhibition of maltase activity of human small intestinal lysate

For inhibition assays, 10 μl of polyphenol compound at a concentration range of 0 to 666.7 μM and 10 μl of maltose solution at the concentration range (2.1-16.7 mM) and 10 μl of the human small intestinal lysate were reacted for 1 h at 37 °C. The reaction was ended by the addition of 170 μl of Tris Glucose-Oxidase (TGO) reagent incubated for an additional 45 min at 37 °C. The optical density was read at 450 nm on the microplate reader (Quezada-Calvillo et al., 1993; Dahlqvist, 1964). A standard curve of glucose was obtained using a range of glucose concentrations (0-400 μg/ml), and the amount of released glucose was calculated.

6.3.6   Inhibition of maltase activity and sucrase activity of MGAM-IP and SI-IP

For the inhibition of maltase activity, 10 μl polyphenolic compound of 0-666.7 μM, 10 μl maltose solution of 2.1-16.7 mM, and 10 μl of MGAM-IP and SI-IP were incubated for 1 h at 37 °C. After the incubation, 170 μl of TGO reagent was added for 45 min at 37 °C. The optical density was read at 450 nm in Synergy microplate reader. The same protocol was repeated by the replacement of maltose solution with the sucrose solution at the concentration range of 2.1-16.7 mM using only SI-IPfor the inhibition of sucrase activity.

6.4   Results

6.4.1   Kinetics and mechanism of inhibition of the maltase activity of human small intestinal lysate

Caffeic and gallic acid exhibited a similar inhibitory potency for the maltase activity of human small intestinal lysate. Caffeic acid inhibited the maltase activity of human small intestinal lysate in a non-competitive heterogeneous manner with Ki_1 and Ki_2 of 65.1 and 67.7 μM (Table 1). Also, the maltase activity of human small intestinal lysate was inhibited by gallic acid in a non-competitive heterogeneous way with Ki_1 and Ki_2 of 71.2 and 72.9 μM (Table 1). (+)-Catechin showed a comparably higher inhibitory effect for maltase activity with a non-competitive heterogeneous inhibition mechanism and Ki_1 and Ki_2 of 14.1 and 87.2 μM (Table 1). On the other hand, EGCG and chlorogenic acid demonstrated competitive inhibition with Ki values of 5.7 μM and 22

μM, respectively (Table 1). Among the compounds tested, EGCG indicated the highest inhibitory potency for the maltase inhibition of human small intestinal lysate.

6.4.2 Kinetics and mechanism of inhibition of the maltase activity of MGAM-IP

The maltase activity of MGAM-IP was inhibited by caffeic acid in a non-competitive heterogeneous way with Ki values of 63.2 and 69 μM (Table 6.1). Gallic acid inhibited the maltase activity of MGAM-IP in a non-competitive heterogeneous manner with Ki_1 and Ki_2 of 48.1 and 64.3 μM (Table 6.1). (+)-Catechin showed non-competitive heterogeneous inhibition mechanism with Ki_1 and Ki_2 of 83.9 and 145.2 μM (Table 6.1). Among the tested compounds, chlorogenic acid and EGCG showed much higher inhibitory potency with Ki values of 7 and 4 μM, respectively (Table 1). Both of these compounds inhibited the maltase activity of MGAM-IP in a non-competitive homogeneous way.

6.4.3 Kinetics and mechanism of inhibition of the maltase and sucrase activities of

SI-IP

Caffeic acid inhibited the maltase activity of SI-IP in a non-competitive heterogeneous manner with Ki_1 and Ki_2 of 130.4 and 132.2 μM (Table 6.2). The sucrase activity of SI-IP was also inhibited in a non-competitive heterogeneous way by caffeic acid with Ki_1 and Ki_2 of 381.9 and 495.8 μM (Table 6.2). Also, gallic acid showed non-competitive heterogeneous inhibition type for both of maltase and sucrase activities of SI-IP. It showed stronger inhibitory effect with Ki_1 and Ki_2 of 37.8 and 262.8 μM on maltase

activity of SI-IP than the sucrase activity of SI-IP (Table 6.2). It had Ki_1 and Ki_2 of 220.5 and 141.7 μM for the inhibition of sucrase activity of SI-IP (Table 6.2). Caffeic and gallic acid showed higher inhibitory potency for maltase activity of SI-IP rather than sucrase activity of SI-IP. On the contrary of caffeic and gallic acid, (+)-catechin demonstrated higher inhibitory potency for the sucrase activity of SI-IP. (+)-Catechin inhibited both of maltase and sucrase activity of SI-IP in a non-competitive manner. It had Ki values of 86 and 129.2 μM for the maltase activity inhibition of SI-IP while Ki value was 34.8 for the sucrase activity inhibition of SI-IP (Table 6.2). While EGCG showed non-competitive heterogeneous inhibition for the maltase activity of SI-IP with Ki_1 and Ki_2 of 25.6 and 83.5 μM, it inhibited the sucrase activity of SI-IP in a non-competitive homogeneous manner with Ki value of 11.1 μM (Table 6.2). However, chlorogenic acid inhibited both of maltase and sucrase activity of SI-IP in a competitive manner with Ki values of 8.1 and 0.7 μM, respectively (Table 6.2). As similar to the (+)-catechin, chlorogenic acid and EGCG showed higher inhibitory potency for the sucrase activity of SI-IP compared to the maltase activity of SI-IP.

6.5   Discussion and Conclusions

Certain phenolics are known to have inhibitory effects on the mucosal α-glucosidases, and there is increasing evidence that dietary phenolic compounds may have an important role in the modulation of glucose release in the small intestine, and subsequent absorption. Although some strong α-glucosidase inhibitors, such as acarbose, are available in the market, researchers still look for other natural compounds exhibiting suitable therapeutic effect for diabetics. Since strong inhibitors largely prevent the

digestion of starch into the glucose, the undigested starch goes to the colon, ferments and may give discomfort to patients. Dietary phenolic compounds, on the other hand, provide partial inhibition of the α-glucosidases and reduce the rate of starch digestion rather than stopping it, and thereby controlling the glucose release in the small intestine.

Most inhibition studies with phenolics have used α-glucosidases from sources other than human small intestinal tissue, such as rat small intestine or yeast. The present study is the first study reporting a clear inhibitory effect of phenolic compounds on human α-glucosidases derived from small intestine tissue. This report provides kinetic data of polyphenol inhibitory potencies on human α-glucosidases.

While our earlier study reported that caffeic acid was not a strong inhibitor with respective IC_{50} values of 0.74 and 0.49 mM for rat intestinal maltase and sucrase activities (Welsh et al., 1989); however, in this study, caffeic acid showed much higher inhibitory potency (lower Ki value) for maltase activity of human small intestinal lysate. A study found that rat intestinal maltase was inhibited by chlorogenic acid in a non-competitive manner with IC_{50} values of 2.99 mM (Ishikawa et al., 2007). Although we reported here only Ki value, the inhibitory effect of chlorogenic acid on maltase activity of the human small intestinal lysate was higher than previously reported ones. Another group found that EGCG had IC_{50} value of 914.8 μmol/L for α-glucosidase of rat intestinal acetone powder (Xu et al., 2013). Another study showed EGCG as a competitive inhibitor for the recombinant human intestinal maltase with IC_{50} value of 20 μM (Nguyen et al., 2012). The inhibitory capacity of EGCG for the maltase activity of human small intestinal lysate was comparable to these results even though Ki value was considered to identify the inhibitory capacity in this study.

Furthermore, our findings indicate there are selective and differential inhibitions of the α-glucosidases that occur in the presence of specific phenolic compounds. For instance, caffeic acid showed similar inhibition constants for the maltase activity of human small intestinal lysate and maltase activity of MGAM-IP, while it showed a higher inhibition constant (lower binding affinity) for maltase activity of SI-IP. A similar case was observed for gallic acid. This suggests that caffeic and gallic acid have a preference for binding the maltase subunit of MGAM. In the case of (+)-catechin, there was higher inhibitory potency for sucrase activity of SI-IP, rather than maltase activity of MGAM-IP and SI-IP. For chlorogenic acid, even though it showed strong inhibitory effect for all tested activities, it was still inhibited sucrase activity of SI-IP in stronger way. Furthermore, chlorogenic acid inhibited both of sucrase activity of SI-IP and maltase activity of both of maltase activity of SI-IP, and human small intestinal lysate, in a competitive way. Similar to chlorogenic acid, EGCG also demonstrated a higher inhibitory potency for all tested activities among all tested compounds. However, it also showed a selective inhibition for the maltase activity of MGAM-IP. Moreover, the similarity in inhibition constants for maltase activity of MGAM-IP and maltase activity of human small intestinal lysate suggests that EGCG targets the maltase subunit of MGAM-IP rather than maltase activity of SI-IP. This study supports earlier work presented in this thesis showing that phenolic compounds inhibit activity of MGAM and SI selectively and differentially, and shows such inhibition in human tissue α-glucosidases. This has not been reported so far in the literature.

In earlier chapters, it was shown that the activities of recombinant individual subunits of MGAM and SI, and as well the α-glucosidases in rat intestinal acetone

powder, are inhibited at different levels by the tested phenolic compounds, and the same patterns were observed using human tissue α-glucosidases. For example, we found that chlorogenic acid and EGCG selectively inhibited the recombinant glucoamylase subunit. In addition, chlorogenic acid showed the highest binding affinity for the recombinant sucrase subunit. Using rat intestinal acetone powder, we found that EGCG and chlorogenic acid showed stronger inhibition capacity, or preference, for maltase and sucrase activity over isomaltase activity.

Results of current study and previous studies indicate that mucosal α-glucosidases are selectively inhibited using natural phenolic compounds. Therefore, the rate of digestion of starches, starch products and sucrose can be modified in the presence of natural phenolic compounds.

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Table 6.1 Inhibition mechanisms and inhibition constants for maltase activity inhibition of human small intestinal lysate and MGAM-IP.

	Maltase activity inhibition of human small intestinal lysate $K_i \pm sd$ (µM)	Maltase activity inhibition of MGAM-IP $K_i \pm sd$ (µM)
Caffeic acid	Non-competitive heterogeneous $K_{i1}= 65.1 \pm 17.7$ $K_{i2}= 67.7 \pm 8.7$ $r^2= 0.971$	Non-competitive heterogeneous $K_{i1}= 63.2 \pm 29.6$ $K_{i2}= 69 \pm 12$ $r^2= 0.923$
Gallic acid	Non-competitive heterogeneous $K_{i1}= 71.2 \pm 30.2$ $K_{i2}= 72.9 \pm 11.3$ $r^2= 0.949$	Non-competitive heterogeneous $K_{i1}= 48.1 \pm 11.1$ $K_{i2}= 64.3 \pm 10.2$ $r^2= 0.973$
(+)-Catechin	Non-competitive heterogeneous $K_{i1}= 14.1 \pm 3.1$ $K_{i2}= 87.2 \pm 30.3$ $r^2= 0.955$	Non-competitive heterogeneous $K_{i1}= 83.9 \pm 32.1$ $K_{i2}= 145.2 \pm 34.1$ $r^2= 0.927$
Chlorogenic acid	Competitive $K_i= 22 \pm 3.3$ $r^2= 0.951$	Non-competitive homogeneous $K_i= 7 \pm 1$ $r^2= 0.990$
EGCG	Competitive $K_i= 5.7 \pm 0.6$ $r^2= 0.965$	Non-competitive homogeneous $K_i= 4 \pm 0.4$ $r^2= 0.980$

Table 6.2 Inhibition mechanisms and inhibition constants for maltase and sucrase activity of SI-IP.

	Maltase activity inhibition of SI-IP $Ki \pm sd$ (µM)	Sucrase activity inhibition of SI-IP $Ki \pm sd$ (µM)
Caffeic acid	Non-competitive heterogeneous $Ki_1=130.4 \pm 30.9$ $Ki_2= 132.2 \pm 50.5$ $r^2= 0.967$	Non-competitive heterogeneous $Ki_1= 381.9 \pm 76.7$ $Ki_2= 495.8 \pm 94.9$ $r^2= 0.978$
Gallic acid	Non-competitive heterogeneous $Ki_1=37.8 \pm 5.4$ $Ki_2= 262.8 \pm 101.7$ $r^2= 0.980$	Non-competitive heterogeneous $Ki_1= 220.5 \pm 72.2$ $Ki_2= 141.7 \pm 20.6$ $r^2= 0.952$
(+)-Catechin	Non-competitive heterogeneous $Ki_1=86 \pm 18.2$ $Ki_2= 129.2 \pm 21.7$ $r^2= 0.969$	Non-competitive homogeneous $Ki= 34.8 \pm 2$ $r^2= 0.984$
Chlorogenic acid	Competitive $Ki=8.1 \pm 1$ $r^2= 0.973$	Competitive $Ki=0.7 \pm 0.1$ $r^2= 0.953$
EGCG	Non-competitive heterogeneous $Ki_1=25.6 \pm 6.2$ $Ki_2= 83.5 \pm 19.8$ $r^2= 0.953$	Non-competitive homogeneous $Ki= 11.1 \pm 1.1$ $r^2= 0.924$

CHAPTER 7. CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

The present study describes the inhibition of α-glucosidic activities of intestinal Maltase-Glucoamylase (MGAM) and Sucrase-Isomaltase (SI) by some selected phenolic compounds previously shown to modulate glucose release. We aimed to show the differential effects of the phenolic compounds on the activities of MGAM and SI to introduce the concept of modulation of starch or sucrose digestion and glucose release using selective and partial inhibition. We proposed to use phenolic compounds, instead of using strong inhibitors which shuts down digestion. Here, selective inhibition means the inhibitor shows different binding affinities for different subunits of MGAM and SI that, when coupled with the appropriate glycemic carbohydrate, this would slow the rate of starch or sucrose digestion. Therefore, the starch or sucrose digestion would still occur, but be completed in the small intestine, and would not cause, or at least reduce, side effects of abdominal discomforts or diarrhea caused by large amounts of carbohydrates entering the colon. There is also the need of showing the underlying mechanism of inhibition of MGAM and SI to control the glucose release in the small intestine. The first study described the selective inhibition of maltase activity of individual mammalian recombinant subunits (C terminal, Ct, and N terminal, Nt) of MGAM and SI by the tested phenolic compounds caffeic acid, gallic acid, (+)-catechin, chlorogenic acid and

(-)-epigallocatechin gallate (EGCG). Among these compounds, EGCG and chlorogenic acid showed selective inhibition of maltase activity of Ct-MGAM. As Ct-MGAM is the most active starch degrading subunit, its inhibition can impact the reduction of the rate of starch digestion and modulate glucose release in the small intestine. Chlorogenic acid highly inhibited maltase activity of Ct-SI (sucrase subunit) in comparison to the other compounds. Thus, this is a promising candidate to decrease the rate of sucrose digestion.

The second study focused on the inhibitory potencies of the above tested compounds on the different activities (maltase, sucrase, isomaltase) of rat intestinal acetone powder. This study differed from the first study in that the inhibition of different activities of entire mixture of MGAM and SI molecules were tested, instead of testing a common activity, maltase activity of individual subunits. This information indicates whether the proposed concept of selective inhibition is valid for the different activities when MGAM and SI are present together. Results showed that the tested compounds, except chlorogenic acid, had selectivity for the rat intestinal maltase activity, rather than sucrase or isomaltase. Similar to findings of first study, EGCG and chlorogenic were the strongest inhibitors for the maltase and sucrase activities of rat intestinal acetone powder. Information obtained from first and second study indicated that selective inhibition of specific activities of the individual subunits of MGAM and SI, as well for different activities of the mixture of MGAM and SI molecules does occur. In a final study on this theme, the same phenolic compounds were tested for inhibition of human small intestinal lysate which includes MGAM and SI, and immunoprecipitated MGAM and SI from the same lysate. Similar to the first two studies, chlorogenic acid demonstrated stronger inhibition of sucrase, or sucrase activity of SI-IP, and also showed strong inhibitory effect

for maltase activity of human small intestinal lysate. Similar to findings of the first two studies, EGCG showed strongest inhibition of the maltase activity of MGAM-IP and the human small intestinal lysate. This shows a preference of EGCG towards maltase activity of MGAM-IP rather than maltase activity of SI-IP. Overall these studies show that similar selective inhibition of activities of MGAM and SI occurs by phenolic compounds independent of the source of biological materials used; however, the binding affinities of these compounds for tested activities of these materials were different. These studies show that natural phenolics are promising candidates to modulate glucose release in the small intestine from starch or sucrose digestion. Moreover, such phenolic compounds can cause a reduction in the rate of starch or sucrose digestion, rather than a stopping of the digestion process. Therefore, the glucose can be released slowly over an extended time that in addition to moderating the glycemic response profile may have additional benefit by triggering the ileal entero-endocrine cells which regulate appetitive response and control nutrient delivery rate from the stomach (gastric empting rate). This may open possibilities to create functional foods with lowered glucose release in the small intestine. The limitation of in vitro inhibiton studies was a lack of knowledge regarding concentration of phenolic compounds required for inhibiting α-glucosidases. Since these phenolic compounds were tested in in vitro assays, the effective concentrations of these tested compounds may be different for in vivo systems.

In this thesis, we showed a selective inhibition of MGAM and SI by phenolic compounds to control glucose release. The other focus of this thesis was to examine gene expression of MGAM and SI by phenolic compounds and observe possible differences in MGAM and SI protein and eventually overall maltase activity. Additionally, caffeic acid,

gallic acid, and (+)-catechin up regulated MGAM mRNA and SI mRNA expression in mouse explants. While differences in the molecular forms of MGAM were observed in Western blots through exposure of phenolic compounds, blots of SI did not show significance change in treating with polyphenols, and overall maltase activity did not change. We can speculate the modification to the α-glucosidases might occur because of the need of effectively digest starch in the presence of polyphenolic inhibitors.

There were some limitations in this study. For instance, the analysis of Western blots particularly those corresponding to MGAM were difficult. Since the transcriptional forms of MGAM have not been well studied, identification of the different forms of MGAM observed on Western blots was somewhat speculative. Future studies are needed to define the underlying mechanism that describes the changes in band proportions.

Future work needs to be done to investigate the selective inhibition concept with phenolic compounds using an in vivo approach. Possible binding sites of these compounds to the individual subunits of MGAM and SI need also to be studied using crystallography methods. Lastly, combinations of polyphenolic compounds should also be tested to determine their potential of interaction during the inhibition process, and, perhaps, transport systems, as well as their effect on starch and sucrose digestion rate.

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