Anti-Diabetic and Anti-Obesity Activities of Cocoa (*Theobroma cacao*) via Physiological Enzyme Inhibition

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Abstract

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Fermentation and roasting of cocoa (*Theobroma cacao*) decrease levels of polyphenolic flavanol compounds. However, it is largely unknown how these changes in polyphenol levels caused by processing affect cocoa's anti-diabetic and anti-obesity bioactivities, such as inhibition of certain enzymes in the body. Polyphenol profiles, protein-binding abilities, presence of compounds termed "oxidative polymers", and abilities to inhibit α-glucosidase, pancreatic α-amylase, lipase, and dipeptidyl peptidase-IV (DPP4) *in vitro* were compared between unfermented bean (UB), fermented bean (FB), unfermented liquor (UL), and fermented liquor (FL) cocoa extracts. Overall, there were significant decreases ($p<0.05$) in total polyphenols, flavanols, and anthocyanins between the two sets of unfermented and fermented cocoa extracts (CEs). All CEs effectively inhibited α-glucosidase (lowest IC_{50} = 90.0 μg/mL for UL) and moderately inhibited α-amylase (lowest IC_{50}=183 μg/mL for FL), lipase (lowest IC_{25}=65.5 μg/mL for FB), and DPP4 (lowest IC_{25}=1585 μg/mL for FB) in dose-dependent manners. Fermentation and roasting of the samples affected inhibition of each enzyme differently (both processes enhanced α-amylase inhibition). Improved α-glucosidase and α-amylase inhibitions were correlated with presence of different classifications of oxidative polymers, suggesting that these compounds could be contributing to the bioactivities observed. Some α-glucosidase inhibition might be due to non-specific protein-binding. Improved DPP4 inhibition was strongly correlated to increased CE degree of polymerization. In conclusion, potential enzyme inhibition activities of cocoa were not necessarily negatively affected by the large polyphenol losses that occur during fermentation and roasting. Additionally, it is possible that more complex compounds could be present in cocoa that contribute to its potential anti-diabetic and anti-obesity bioactivities.

General Audience Abstract

Anti-Diabetic and Anti-Obesity Activities of Cocoa (*Theobroma cacao*) via Physiological Enzyme Inhibition

Caroline Mary Ryan

During the manufacture of various cocoa products, cocoa (*Theobroma cacao*) beans undergo fermentation and roasting. However, during these processes, the naturally high levels of polyphenolic compounds in cocoa are significantly decreased due to high temperatures, presence of oxygen, etc. It is believed that some of the polyphenols in cocoa have the ability to interact with and inhibit the activity of various enzymes in the body. The enzymes explored in this research are those that help to digest carbohydrates and lipids and maintain normal blood glucose levels. Inhibition of these enzymes can delay the breakdown of carbohydrates and lipids, thus potentially reducing fat accumulation and increasing satiety, as well as reducing large spikes in blood glucose levels. These effects have the potential to aid in the prevention and/or amelioration of Type-2 Diabetes and obesity. Since it is widely unknown how the changes in cocoa's polyphenol levels due to fermentation and roasting affect its ability to inhibit these enzymes, this research strove to explore these concepts and draw correlations between them. Overall, large losses of polyphenols were observed due to fermentation and roasting of cocoa samples. However, these losses did not always demonstrate decreases in inhibition abilities. In some cases, fermentation and/or roasting showed to improve enzyme inhibition. Other compositional characteristics of the cocoa were also measured and some were correlated with improved enzyme inhibition. This research is one of the first studies exploring how compositional changes due to processing affect (or potentially enhance) some of cocoa's potential health benefits.

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Chapter 1:
Introduction

As of 2012, over 30% of adults and 17% of children in the United States are obese.[1] Obesity is a growing concern in the world today since it is strongly related to the onset or worsening of other serious conditions such as cardiovascular disease, diabetes, stroke cancer, and arthritis.[2, 3] It is expected that by 2030, diabetes will be the 7th leading cause of death and that 366 million people in the world will be diagnosed with the disease, a 114% increase from 2000.[4, 5] About 90-95% of all adult diabetes diagnoses are Type-II, with which, individuals are able to secrete the glucose uptake-regulating hormone, insulin, but their bodies are unable to utilize it properly.[6] Type-II diabetes is strongly related to obesity in that the excess energy that cannot be regulated by insulin is often stored as fat in the body.

Recently there has been an interest in utilizing natural products as a potential complimentary strategy to prevent and/or ameliorate symptoms of these diseases due to high levels of polyphenol compounds. In particular, cocoa has been under examination for the prevention of Type-II diabetes and obesity,[7, 8] as it contains the highest amount of flavanol compounds (a subclass of polyphenols) compared to any other food.[9-11] During processing of cocoa, these levels of polyphenols decrease due to conditions of high temperatures, presence of oxygen, changes in pH, etc.[12-14] Therefore, we strove to look deeper into this concept and determine if these changes in polyphenol levels affect cocoa's potential health benefits.

While the mechanisms by which cocoa may exert bioactivities remain unknown, research has suggested that these flavanol compounds (including (−)-epicatechin, (+)-catechin, and their polymerized procyanidins) have the abilities to interact with and inhibit activity of certain physiological enzymes.[7, 15] Our research aimed to determine potential inhibition of the carbohydrate-digesting enzymes α-glucosidase and pancreatic α-amylase, the fat-digesting enzyme, pancreatic lipase, as well as the blood glucose-regulating enzyme, dipeptidyl peptidase-IV (DPP4). By inhibiting α-glucosidase, α-amylase, and lipase, carbohydrates and fats are digested over a longer period of time, thus potentially alleviating spikes in blood glucose, reducing fat accumulation, and increasing satiety. Inhibition of DPP4 allows for glucose-regulating hormones to proliferate longer in the gut, thus promoting insulin secretion and maintaining normal blood glucose levels.

The factors or polyphenol characteristics that are responsible for these bioactivities are largely unknown. Previous researchers have drawn conclusions on the effects of things such as compound size, concentration, and structure on enzyme inhibition.[16-18] This lead to another goal of this research: to characterize abilities of cocoa to bind proteins, which could then potentially be correlated to their abilities to inhibit enzymes. Additionally, we were lead to believe that there could be other compounds besides flavanols in cocoa contributing to its bioactivities.

In order to achieve the goals of this research, the following specific aims were proposed and tested:

1. Characterize the polyphenolic composition of four different cocoa extracts including total polyphenol, flavanols, anthocyanins, and mean degree of polymerization.
2. Assess the effectiveness of the cocoa extracts as inhibitors of α-glucosidase, pancreatic α-amylase, pancreatic lipase, and dipeptidyl-peptidase-IV (DPP4).
3. Measure the presence of oxidative polymer compounds and protein binding abilities of the cocoa extracts.
4. Determine if there is a correlation between enzyme inhibition and polyphenolic profile, presence of oxidative polymers, and/or protein binding abilities.

Initially we hypothesized that the cocoa products that have been fermented and/or roasted would have lower levels of polyphenols and would be less effective enzyme inhibitors. Overall, we thought that effectiveness of enzyme inhibition would be proportional and correlated to polyphenol/flavanol concentration (samples with higher polyphenol levels would be more effective inhibitors due to the higher concentration of these compounds).

We believe this research is both novel and important because we are one of the first groups to examine cocoa's inhibition of α-glucosidase and DPP4 as well as potentially correlate enzyme inhibition abilities to protein binding and presence of another classification of compounds. This preliminary study will hopefully open many doors for more specific classification and quantification of these new compounds as well as inspire further research on the abilities of cocoa to help prevent Type-II diabetes and obesity.

Chapter 2

Review of Literature

Polyphenols. Polyphenols are compounds composed of many phenol subunits (**Figure 2.1**). They are secondary plant metabolites, meaning that they are not required for human or plant growth and development, and are often found in various plants with higher concentrations of polyphenols found on leaves and outer areas of the plants.[19, 20]

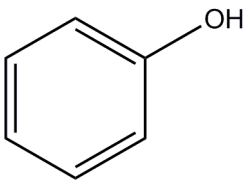


Figure 2.1. *Basic phenol structure*

Polyphenolic compounds are very commonly found in fruits, vegetables, tea, and numerous other plant-derived food products such as wine, extra virgin olive oil, chocolate and other cocoa products.[21, 22] To date, approximately 8000 phenolic compounds have been identified and can be divided into at least ten different groups depending on their structure, including phenolic acids, flavonoids, lignans, stillbenes, polyphenolic amides, and other non-flavonoid polyphenols.[19, 21, 23, 24] Most polyphenols are found in a glycosidic form yet vary in the number and position of the sugar units, providing a unique identity to each compound.[23] Since the structures can be extremely different, this is why there are numerous subclasses of polyphenols as described above. Of these subclasses, flavonoids are the most abundant polyphenols in the human diet with over 4000 compounds identified,[21, 25, 26] which are discussed further in the following section. The remaining subclasses are not as large as the flavonoid group, yet consist of other compounds that are commonly found in foods as well. Just as polyphenols vary in number and position of sugar units, they also differ in compound size, chain length, number of subunits, positions of linkages, and presence of substituent units.[27] These are few amongst many reasons as to why polyphenol classification can be difficult, yet never-ending.

Polyphenols are highly reactive compounds and the different subclasses possess varying stabilities, bioavailabilities, and functions.[23, 27] Polyphenols can provide benefits to the plants themselves, supporting cell walls and acting as defense mechanisms to environmental stresses,[28]

as well as providing benefits *in vivo* upon ingestion of the compounds. The most commonly known bioactivity of polyphenols is their antioxidant capabilities. These compounds can act as electron donors to scavenge free radicals, fighting against oxidative stress caused by these reactive oxygen species (ROS).[22, 23, 29] These actions not only help improve overall cell proliferation, but also contribute to other beneficial bioactivities. There is a large body of evidence and review on polyphenols in foods promoting heart health via anti-inflammatory, anti-atherosclerotic, cholesterol-lowering, and blood pressure-reducing mechanisms.[21, 22, 30] In addition to these activities, polyphenols have also shown protection against neurodegenerative diseases, anti-cancer, metal chelation, improvement of endothelial function, anti-diabetic, and other health-promoting effects.[22] These functions and variations in structure demonstrate just how diverse these compounds are biologically, which is why it is important to focus in on specific subclasses of polyphenols and their benefits to health.

Flavonoids. As stated above, flavonoids are the largest, most common, and most widely distributed class of polyphenols[31]. They are hydroxylated phenolic compounds each one consisting of a 15-carbon core structure with two benzene rings and one heterocyclic pyrane ring (**Figure 2.2**).[32]

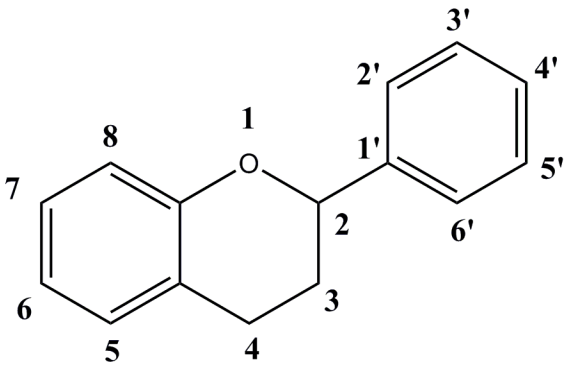


Figure 2.2. *Skeletal structure of a flavonoid*

Flavonoids, like all polyphenols, are phytochemicals; meaning that they must be obtained through the diet as humans and animals cannot synthesize them on their own.[26] There are about 13 classes of flavonoids including anthocyanins, flavanols, flavanones, flavones, flavonols, and isoflavones (**Figure 2.3**).[19, 25, 31].

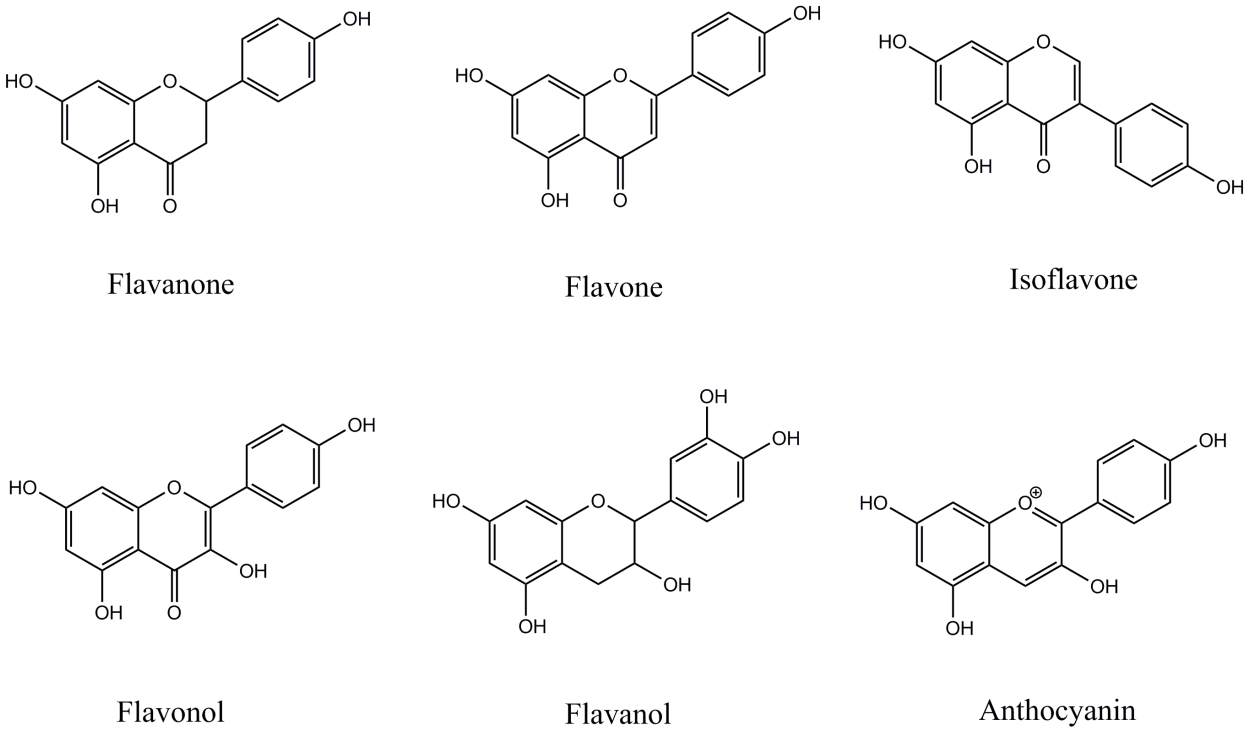
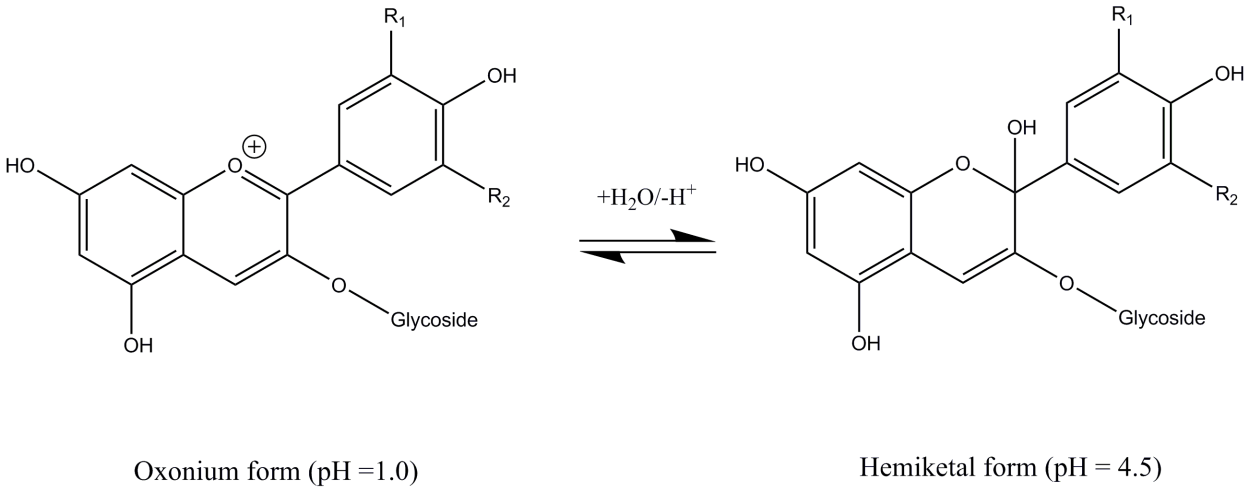


Figure 2.3. *Six main classes of flavonoid compounds*

These compounds are often found in foods such as red wine, fruit juice, coffee, beer, tea, soy, citrus fruits, berries, cocoa, and onions.[31, 33, 34] Flavonoids are some of the most important pigment compounds in plants aside from chlorophyll and carotenoids, contributing to the color of the plant itself and, in some cases, its resulting food product.[31, 35] Once ingested, absorption of flavonoids in the body varies from very well absorbed to little or no absorption whatsoever as it depends on factors such as the compound size, structure, solubility, stability in pH, etc.[32] Therefore, it is thought that since these compounds are often not efficiently absorbed by the intestine, the main site of their activity is in the lumen of the gastrointestinal tract.[36] Although absorption may be poor, flavonoids have shown many different biological activities including antioxidant, hepatoprotection, anti-bacterial, anti-inflammatory, anti-viral, anti-cancer, and inhibition of LDL oxidation.[37] Once again, the diversity of these compounds is demonstrated by their structure-dependent activities. Within the flavonoid classification, the flavanol and

anthocyanin compounds are of the most importance to this research, both of which will be discussed in subsequent sections of this literature review.

Anthocyanins. Anthocyanins are the glycoside forms of anthocyanidins and are the most important group of water-soluble plant pigments, often contributing color to plants.[19] Anthocyanins are commonly found in products such as berries and grapes, and thus contribute to the red color of wine.[19, 38] Monomeric anthocyanins undergo reversible structural transformations at different pH values: present as the red-colored, oxonium form at pH 1.0 and as the colorless hemiketal form at pH 4.5 (**Figure 2.4**).[39] Four anthocyanin forms exist based on structure and absorbance differences; however, this research was focused on the two forms shown below. Differences in absorbance at these pH levels can then be measured and related to concentration of monomeric anthocyanins.



Oxonium form (pH =1.0) Hemiketal form (pH = 4.5)

Figure 2.4. *Representative structural change reaction from the oxonium and hemiketal anthocyanin forms between pH 1.0 and 4.5*

However, during various fermentation processes, such as wine fermentation, anthocyanins are hydrolyzed to anthocyanidins and then polymerize with catechins and other flavonoid compounds to form complex "tannin" structures.[12] In the case of wine fermentation, these tannin structures that are formed are often known as polymeric pigments, which also contribute to the dark colors of wine.[40] Anthocyanins are also found in lower concentrations in other natural products, such as cocoa, of which anthocyanins contribute to about 4% of total

polyphenols.[12] However, previous studies have shown that cocoa fermentation significantly reduces levels of anthocyanins, some losses resulting in the formation of larger, brown polymeric compounds.[12, 41]

Consumption of anthocyanin-rich foods has been reviewed and reported to help prevent or alleviate chronic diseases such as cardiovascular disease, cancer, and hyperlipidemia.[42] Therefore, it is of great interest to measure concentrations of these compounds to potentially find a correlation between anthocyanins and other measured health benefits. While the majority of this research was focused on flavanols and oxidative polymers (as discussed in subsequent sections) as potentially bioactive components of cocoa, measurement of anthocyanins was of interest as well, to determine if this type of compounds was also health-beneficial.

Flavanols. Flavanols (also known as flavan-3-ols) are the most common group of flavonoids consumed in the U.S. diet, found in foods such as red wine, tea, grapes, chocolate, apples, grains, and other fruits and vegetables.[43, 44] The monomeric forms of flavanols are known as catechins. These compounds can be found in trans (catechin) or cis (epicatechin) conformations with both (+) and (–) isomers.[23, 45] Procyanidins are oligomers and polymers formed by the joining of multiple monomeric subunits: 2-5 subunits for oligomeric procyanidins and >5 subunits for polymeric procyanidins (classification of oligomers versus polymers may vary).[45] Procyanidins are often identified based on characteristics such as their structure and degree of polymerization, which is the number of monomeric subunits within the polymeric structure.[8] Many types of procyanidins exist based on the linkages between monomers; most commonly identified are A- and B-type procyanidins. Cocoa procyanidins are mostly B-type procyanidins, in which the monomeric subunits are usually linked via C4→C8 linkages.[46] **Figure 2.5** below shows the structures of monomeric (+)-catechin, (–)-catechin, (–)-epicatechin, dimeric procyanidin B2, and a pentamer procyanidin (DP=5), flavanols commonly found in cocoa and other similar food products.

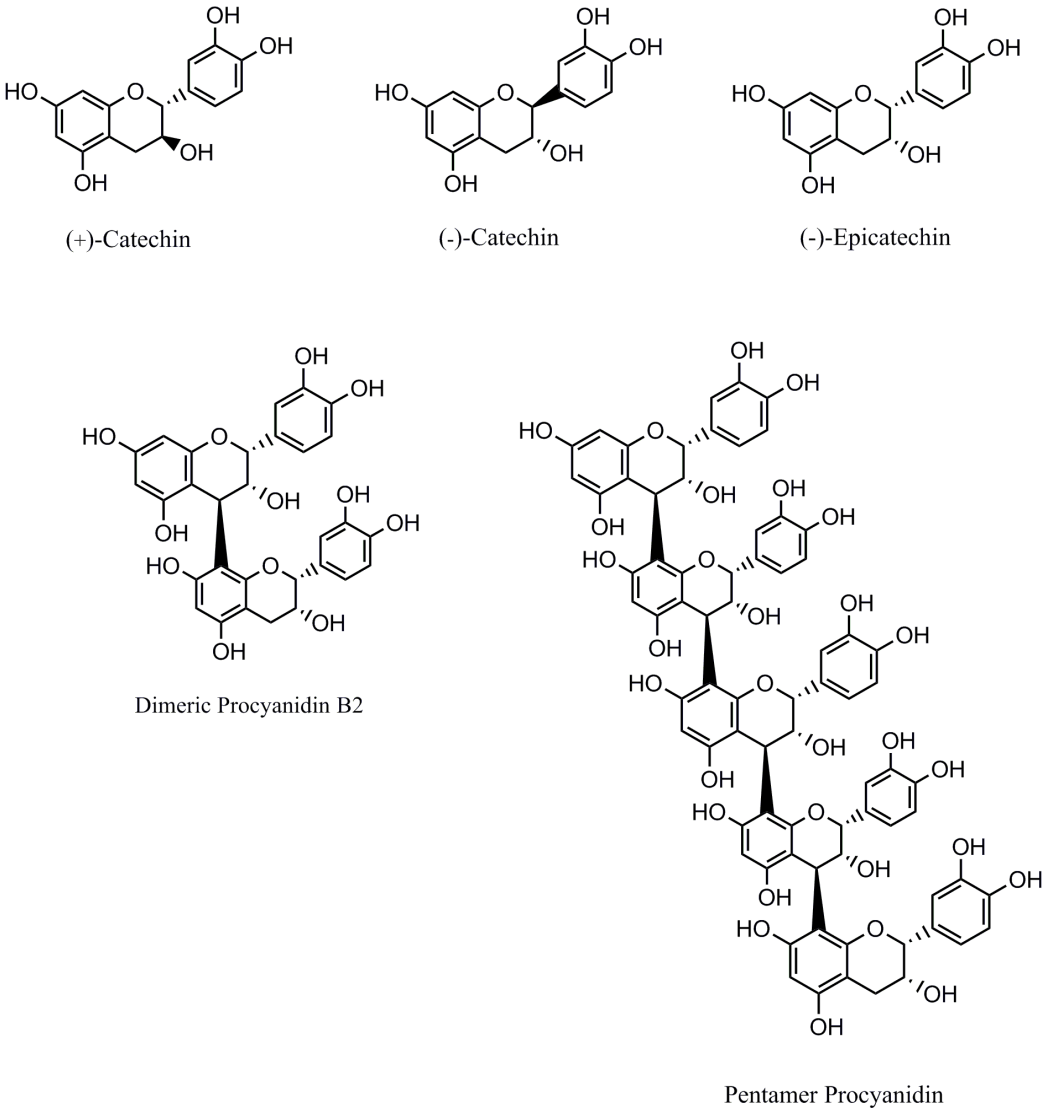


Figure 2.5. *Representative structures of monomeric and oligomeric flavanols commonly found in cocoa*

Flavanols are known to have antioxidant, anticarcinogenic, cardioprotective, antimicrobial, antiviral, and neuroprotective activities.[43] Studies have implied that flavanols are one of the more effective phenolic groups in reducing the risk of coronary heart disease.[47] A recent study by Litterio *et al*[48] observed increased nitric oxide bioavailability in rats fed high-sucrose diets supplemented with (–)-epicatechin, thus preventing increases in blood pressure and hypertension, demonstrating the heart-health benefits of flavanol supplementation *in vivo*. Flavanols are mainly absorbed in the small intestine with monomers absorbed at higher rates than dimers, trimers, and other polymerized flavanols.[11, 49, 50] While absorption is observed, larger

compounds have low bioavailability; therefore, it is believed that one of the main locations of their activity is in the lumen of the gastrointestinal tract.[51] Bioavailability related to cocoa flavanols is discussed in further detail in a separate section below.

Flavanols in Cocoa. Cocoa is a polyphenol-rich food and contains the highest amount of flavanols compared to any other food on a per-weight basis.[9-11] The polyphenols are located in storage or pigment cells of the cotyledons (cocoa nibs) and diffuse out of the beans through water release.[52] On a dry weight basis, cocoa beans are approximately 12-18% polyphenols, which contribute to both the color and flavor of resulting chocolate products.[19, 53] The main polyphenol in cocoa is (–)-epicatechin (comprising about 35% of total polyphenols), followed by (+)-catechin and dimers and trimers of these monomers.[12, 53-56] Procyanidins B2 and B5 are the major dimers present in cocoa and oligomers mostly consist of (–)-epicatechin subunits.[56, 57] The monomers comprising oligomeric flavanols are most commonly bound by 4→6 or 4→8 linkages, which in turn determines structure and function of the compounds (**Figure 2.6**).[56]

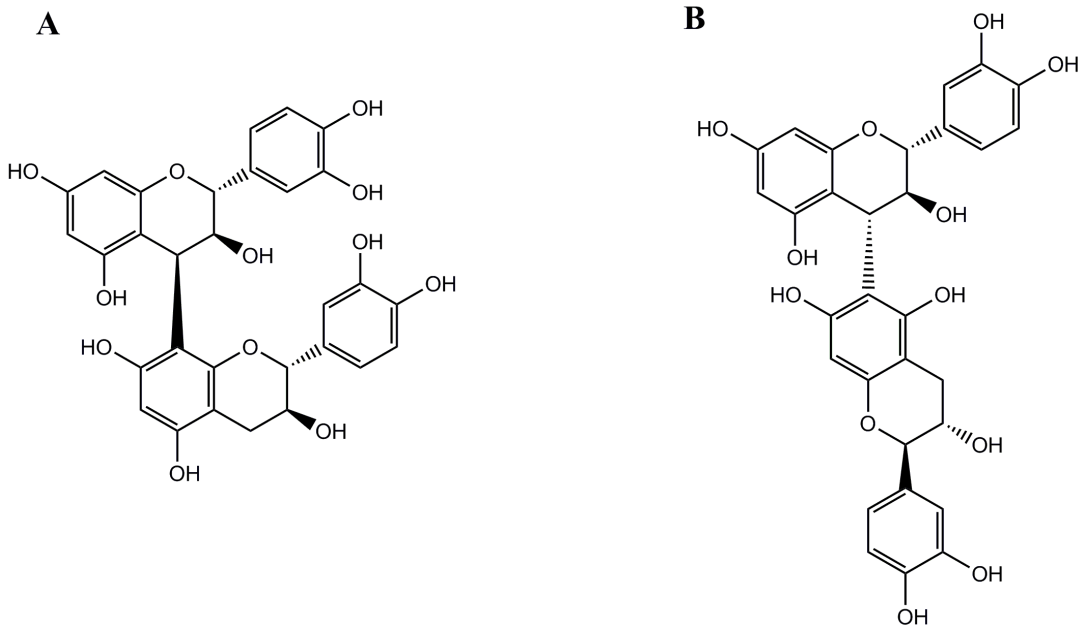


Figure 2.6. *Dimeric procyanidins with monomers joined by* ***(A)*** *(4→8) linkage and* ***(B)*** *(4→6) linkage*

Polyphenol content defines overall flavor and some quality characteristics of the cocoa. For example, high levels of flavanols and other polyphenols in cocoa have shown to contribute a bitter flavor and astringent nature to the nibs.[58-60] However, chemical reactions such as oxidation, polymerization, and complexation of the polyphenols with proteins have the ability to decrease astringency of the cocoa beans.[58]

Numerous studies have been performed and have shown that dietary supplementation with cocoa, cocoa extracts and/or flavanol-rich chocolate products may reduce blood pressure, hyperglycemia, insulin resistance and other symptoms of diabetes and obesity in animal models[8, 61-64] and human subjects.[65-69] We believe that these health benefits are largely due to flavanol bioactivities, which is why these compounds are of major focus in cocoa and its resulting chocolate products. However, cocoa polyphenol contents can vary based on aspects such as bean origin and fermentation practices on the farms.[53, 56, 70, 71] Additionally, processing induces different effects on polyphenol levels in cocoa, which will be discussed in detail in subsequent sections of this literature review. It is important to understand these variations and changes during processing to determine if and how flavanol bioactivities are affected. While the mechanisms of flavanol activities still remain unclear, it is commonly accepted that these are highly beneficial compounds to human health.

Cocoa Processing. The scientific name for cocoa is *Theobroma cacao*, roughly meaning "food of the gods" from Greek.[62] Cocoa beans grow in pods on the *Theobroma cacao* tree, which are found in areas ± 20° of the equator where the climate is warm and moist, allowing year-round growth and production.[12] From 2009-2012, an average of 3.5 million tons of cocoa was produced per year on small cocoa farms in major producing countries such as the Ivory Coast, Ghana, Indonesia, Brazil, Nigeria, Cameroon, Malaysia, and Ecuador.[72, 73] Upon harvest, the cocoa pods are cut open, exposing about 20-50 beans covered in a white mucilage, which are then laid out to undergo fermentation and drying steps, which will be discussed further in the following section as they are crucial steps in cocoa processing. After the beans are dried, they are usually exported to chocolate manufacturers for use. **Figure 2.7** below illustrates the process flow of cocoa and chocolate production discussed in this section.

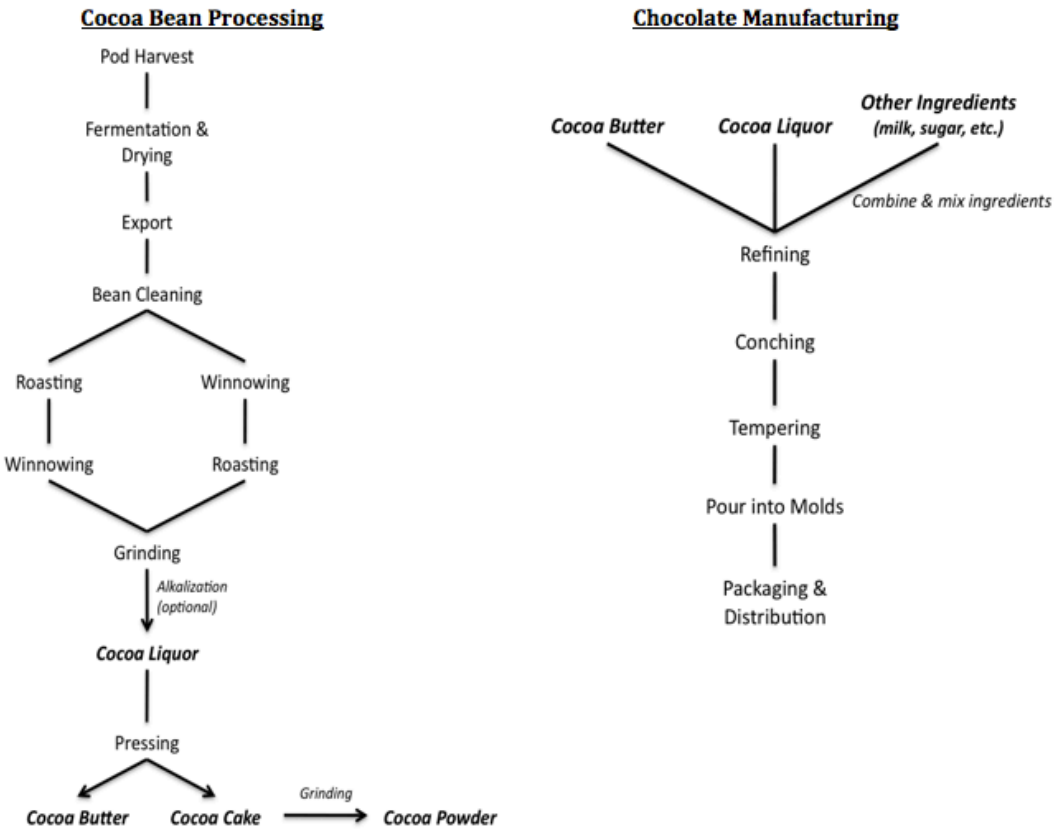


Figure 2.7. *Process flow diagram of cocoa and chocolate production*

Upon arrival at the manufacturing facility, the beans are first cleaned and then either the whole bean or the cocoa material inside (nibs) undergo a roasting step.[12] If just the nibs are to be roasted, the beans first are winnowed, which is cracking and air-blowing off the bean shells to obtain the cocoa inside.[73] The order of the winnowing and roasting steps is interchangeable, depending on the manufacturer's preference. Roasting is another crucial step in the production of chocolate and cocoa products, as it facilitates in development of the flavor precursors formed during fermentation.[12] This step varies in both length of time and temperature it is performed at. Roasting temperature typically ranges from 120 to 150°C and can take anywhere from 5 to 120 min.[12] This step not only helps define the flavor of the resulting cocoa, but also helps to dry out the beans further, making them more brittle and easier to be ground up, which is the next step in cocoa processing. Roasting can also be important for pasteurizing the beans in order to reduce food safety risks. The roasted nibs are ground at high speed into what is known as "cocoa liquor", which is a fluid paste that is solid at room temperature containing all cocoa solids.[73] At

this point, manufacturers can choose to perform a "dutching" (also known as alkalization) step to the cocoa. This would involve the addition of an alkali solution to the cocoa, which in turn reduces the acidity of the cocoa, gives it a darker color, and helps define its chocolate flavor. The cocoa liquor is then pressed by large hydraulic presses and its components are separated into cocoa butter and cocoa cake, which is then further ground into cocoa powder.[73]

Once the cocoa components have been processed and separated, chocolate production can begin with the combination and mixing of cocoa butter, cocoa liquor, and other ingredients such as milk, sugar, flavorings, etc. The resulting liquid can then be refined, during which it is smoothed out by large rollers, resulting in smaller particles and better mouth feel.[12] Next would be a conching step, which is continuous stirring and agitation of the cocoa liquid at about 50-60°C for hours to days, resulting in finer crystallization of the sugars that helps improve overall chocolate quality. The chocolate is then tempered (continuous heating and cooling to provide a glossy finish), dispensed into molds, cooled, packaged, and distributed by the company.

While this research mainly focused on the fermentation/drying and roasting steps of this process, it is important to understand the subsequent steps and how the bioactive components in cocoa are affected so that we can potentially adjust the processes to take these changes into account. Cocoa beans initially have a high polyphenol concentration; however, processing of the beans (including steps such as roasting and alkalization) reduces that content >10x, decreasing their antioxidant capabilities and other bioactivities.[13, 14, 58, 74, 75] It is believed that the steps of the process with high temperature and/or oxygen presence account for these changes in polyphenol content.[12] This challenge is initially what led us to develop the concept of this research: how does processing (particularly fermentation/drying and roasting) affect the polyphenol content of cocoa and how do these changes affect its bioactivity in relation to diabetes and obesity. Research has been performed previously studying how alteration of different processing aspects, such as pulp preconditioning and pod storage, can help with polyphenol losses.[76, 77] Recently, one study by Hu *et al*[78] examined the effects of puffing the cocoa beans using heat and pressure versus conventional roasting on polyphenol content and antioxidant capacity of cocoa. They found that puffing of the cocoa at certain pressures resulting in cocoa with higher total polyphenols, flavonoids, and antioxidant capacity than the roasted cocoa, with no significant differences in sensory attributes.[78] This study helps pave the way of future research to be done, altering the

cocoa production process to maintain high levels of polyphenols without sacrificing the sensory components that consumers have grown accustomed to.

Cocoa Fermentation and Drying. As stated above, the fermentation and drying steps of cocoa processing are crucial for the resulting products. Much research has been performed on the microbial and chemical changes that take place during this step and how it affects the cocoa. Essentially, the cocoa beans are sterile until the pods are cut open and contaminated by sources such as workers' hands, knives, baskets, pulp in boxes, etc.[73] It is this contamination by natural microflora that contributes to the microbial fermentation process. The beans are typically heaped into piles or placed into trays or bins, covered with leaves, mats, etc. and let sit for about 5-6 days.[12] During this time, the temperature rises to about 45-50°C which kills the cocoa beans, known as "bean death".[73, 79] To begin, yeasts and acetic acid bacteria break down the mucilage around the beans, producing ethanol and lactic acid bacteria and giving off heat.[73, 80] Around day 3, the ethanol is oxidized, resulting in acetic acid, an important metabolite causing fermentation to end, which is further oxidized to carbon dioxide and water.[73, 81] It is this production of ethanol and acetic acid under anaerobic conditions that is thought to cause the compositional changes in the cocoa beans, causing the polyphenols to diffuse out with the liquid mucilage and undergo oxidation by polyphenol oxidase (PPO), forming highly polymerized, insoluble compounds.[12, 53, 73] Compounds commonly found in cocoa, such as catechins, epicatechins, and anthocyanins, are substrates for PPO, and therefore contribute to this oxidation step.[82] In addition to oxidation of these compounds, important flavor precursors are formed during fermentation, which develop later on during roasting.[83] The flavor compounds can develop as quickly as within 20 hours of fermentation and continue to develop under these conditions.[79] After fermentation is complete, the coverings are removed from the cocoa beans and they are allowed to dry. Aerobic oxidation and non-enzymatic browning takes place within the cocoa nibs, further defining flavor and color, until the beans obtain a moisture content of 5-7% to avoid mold growth.[12, 21, 84]

Even though these steps are extremely important for chocolate quality, fermentation has shown to significantly decrease total polyphenol contents.[13, 29, 72, 85] Epicatechin decreases mostly during days 2 and 3, resulting in about 10% of its original concentration, and procyanidins have shown 3-5 fold decreases throughout the entire fermentation process.[12, 76] These losses can be contributed to oxidation by PPO, which has optimal activity at pH 6 and 35.5°C, and has shown

to remove over 80% of total polyphenols in 15 min.[80, 86] Monomeric flavanols can also isomerize and polymerize due to fermentation conditions, which affects overall concentration and composition of polyphenols.[60, 87-89] Anthocyanins are either completely lost during fermentation and drying or are oxidized by PPO, ultimately polymerizing with other flavonoids to form large tannin structures.[12, 29, 73, 79] Successful fermentation is very important and is dependent on factors such as temperature, pH, and aeration; or else the resulting cocoa may be under-fermented, and bitter.[58, 90] Unfermented cocoa may be produced and utilized in some cases such as this research. *Lavado* is the term used to describe unfermented cocoa that has undergone subsequent steps of the process (such as roasting and grinding). Unfermented cocoa is not often used in the industry since the high amount of polyphenols imparts a strong bitterness to the cocoa.[91]

The challenge that arises is how to produce cocoa beans with high amounts of bioactive polyphenols, yet still obtain the ideal flavor precursors brought about by fermentation. There have been studies done to test how processing location, pulp conditioning, and fermentation conditions affect polyphenol content, and they all have concluded that this step of the process still has the strong ability to decrease polyphenols.[53, 76, 81, 92] One study performed by Tomas-Barberan *et al*.[82] used different time-temperature heating conditions to evaluate the impact of browning from PPO on cocoa polyphenols. They found that blanching the beans at 95°C for 5 min resulted in the lowest enzymatic browning, and concluded that altering processing conditions has the potential to "enhance flavonoid composition in cocoa powder".[82] This study especially demonstrates the potential to alter the cocoa process in order to produce cocoa beans high in polyphenols while maintaining proper sensory characteristics. If it were possible to protect these bioactive components while still being able to produce the chocolate that consumers are familiar with, it could impact the cocoa and chocolate industry in highly beneficial ways.

Flavanol Bioavailability. Polyphenols such as flavanols are often identified as xenobiotics within a living organism, meaning that the organism cannot produce and does not expect the compound to be present in the living system. This affects the extent to which flavanols are absorbed into the bloodstream and utilized *in vivo,* a characteristic known as bioavailability. Larger flavanols have poor bioavailability, which limits the effectiveness of their health-beneficial activities in the body and poses a challenge to polyphenol scientists.

In the small intestine, smaller compounds are more readily absorbed, whereas larger compounds move into the large intestine.[11, 30, 93] It is estimated that about 90-95% of ingested polyphenols are not absorbed in the small intestine, thus passing into the large intestine.[94] Those larger, unabsorbed compounds are then subjected to the microbiota in the colon, which transforms them into compounds that are more readily absorbed.[95] B-type procyanidins first undergo C-ring opening by intestinal microbiota followed by various reactions such as lactonization, decarboxylation, oxidation, and many others to form hydroxyphenylvalerolactones (γ-valerolactones) and valeric acids.[95, 96] These metabolites then undero dehydroxylation and further reactions to form phenolic acids, which are then absorbed into the bloodstream through the colon, metabolized in the liver, and often excreted in the urine.[95] **Figure 2.8** shows a basic mechanism by which epicatechins are metabolized and biotransformed by colonic microbiota.[97]

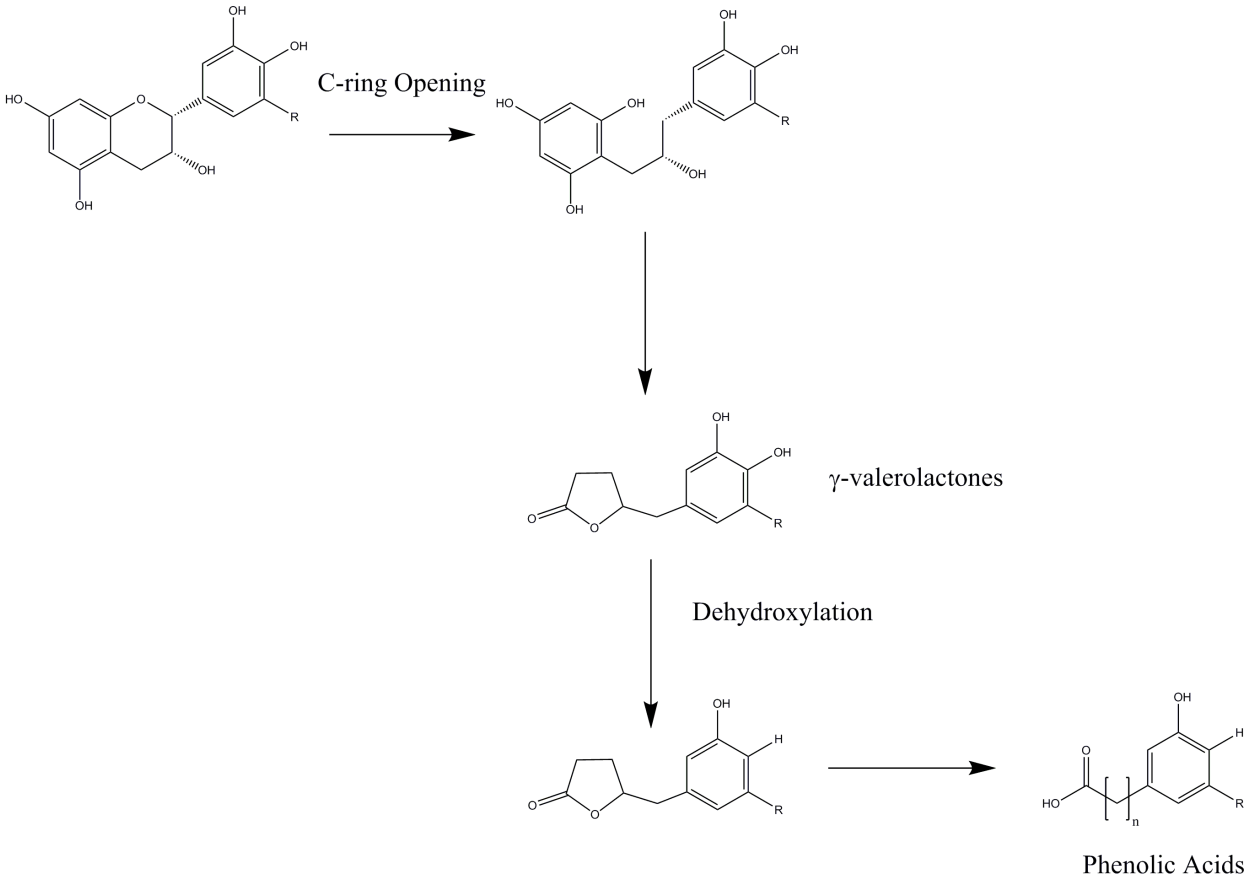


Figure 2.8. *Representative microbial biotransformation of epicatechins in the colon*

Absorption of flavanols depends on factors such as compound size/polymerization, concentration, and stereochemistry.[8, 19, 92] Additionally, flavanols can easily bind to proteins in the body, which may be another reason for their poor absorption.[93] It is commonly accepted that the larger the flavanol, the less it will be absorbed into the bloodstream. According to Cooper *et al.*[92], smaller polyphenols have a larger chance of reaching its target organ than those that are larger. About 22-55% of epicatechin and catechin are absorbed in the small intestine, whereas less than 0.5% of dimers and trimers are absorbed there.[21] If the larger compounds are to be absorbed at all, they must enter the large intestine and be biotransformed by microbiota before entering into the bloodstream.[95] In one study conducted by Gonthier *et al.*[98], rats were fed diets with catechin, procyanidin B3, procyanidin C2, and polymeric procyanidin and metabolism of each compound was measured. Approximately 25.7% of the ingested catechin was excreted in the forms of intact catechin and its 3'-O-methylated derivative formed in the small intestine and liver, demonstrating the good absorption of monomers. On the other hand, as the DP of the remaining procyanidins increased, the amounts of derivatives measured decreased, suggesting that the larger compounds were not biotransformed and absorbed as efficiently.[98]

Stereochemistry and chirality also may affect flavanol bioavailability. It has been reported that (+)-catechin is about 10x more absorbed that its stereoisomer (–)-catechin.[92] In a recent study performed by Ottaviani *et al.*[99], healthy adult males were instructed to consume a cocoa-based dairy drink containing either (–)-epicatechin, (–)-catechin, (+)-epicatechin, or (+)-catechin and their plasma and 24-hour urine were analyzed. They ultimately concluded that absorption of the compounds was as follows: (–)-epicatechin > (+)-epicatechin = (+)-catechin > (–)-catechin as measured in the blood plasma 2 and 4 hours after consumption.[99] While there was no distinct isomeric form that clearly predominated, it was clear that stereoisomerism does, in fact, affect flavanol absorption, and thus its bioavailability.

In a recent study, it was shown that age also influences flavanol absorption. Margalef *et al.*[100] administered grape seed polyphenol extract to rats of varying ages and found that the older rats had reduced intestinal absorption and phase II metabolism of the polyphenol extract with higher levels of metabolites remaining in the colon. The researchers concluded that there may be other factors besides the polyphenols themselves, such as consumer age, that affect flavanol absorption and bioavailability.

Poor absorption and bioavailability are constant challenges with many cocoa polyphenols, providing difficulty in determining bioactive mechanisms and health benefits. According to Manach *et al*.[101], cocoa polyphenols have low C_{max} values in the bloodstream, short half-lives, and are rapidly excreted, all factors contributing to their poor bioavailability. In a study by Baba *et al*.[102], five healthy male subjects were given a chocolate or cocoa product to consume and their plasma and urine were collected and analyzed for epicatechin and its metabolites. Excretion of epicatechin was about 29.8% and 25.3% for the chocolate and cocoa, respectively.[102] This study demonstrated good absorption of cocoa monomers *in vivo*. However, size and absorption are not necessarily always proportional in relation to bioactivity, meaning that in some cases, while smaller compounds are more readily absorbed, this does not necessarily mean that they are the most bioactive. Upon comparing bioactivities of cocoa monomers, oligomers, and polymers *in vivo*, Dorenkott *et al*.[8] found that oligomeric cocoa procyanidins had higher bioactivity in the prevention of obesity, insulin resistance, and impaired glucose tolerance during high-fat feeding.

Along the same lines, polyphenolic compounds are absorbed and utilized in various ways, resulting in high variation of bioavailabilities and bioactivities. Since these compounds are digested and absorbed differently *in vivo*, the metabolites formed during these processes are what contribute to these biological functions in a lot of cases, making it more difficult to isolate and identify causes of bioactivity. Variations in bioavailability cause much debate in the consumption and activity of polyphenolic compounds. For example, although one type of polyphenol may be the most consumed in the diet, that does not necessarily mean that it is the most bioactive as it may have very low bioavailability even in large concentrations. This provides the opportunity for researchers to bridge these gaps in knowledge and identify bioactive components that display high bioactivity and health benefits.

Obesity and Diabetes in the United States. As of 2012, over 30% of adults and 17% of children in the United States are obese[1]. Obesity is affected not only by level of energy expenditure, but also by energy intake and metabolism. Excess energy that is consumed is stored as fat in the body, resulting in an increase in both size and number of fat cells[18]. Obesity is a growing concern in the world today as it affects many people and because it is strongly related to

the onset or worsening of other serious conditions, such as cardiovascular disease, diabetes, stroke, cancer, and arthritis.[2, 3]

Diabetes is a disease where individuals have abnormally high levels of glucose in their blood. It is expected that by 2030, diabetes will be the 7th leading cause of death and that 366 million people in the world will be diagnosed with the disease, a 114% increase from 2000.[4, 5] Diabetes is classified as either Type-I or Type-II. With Type-I diabetes, individuals are unable to secrete the hormone insulin, which helps glucose absorb into cells. However, about 90-95% of all adult diabetes diagnoses are Type-II, with which, the individuals are able to secrete insulin, but their bodies are unable to utilize it properly.[6] Type-II diabetes is strongly related to obesity in that the excess energy that cannot be regulated by insulin, is then often stored as fat in the body.

Obesity and Type-2 Diabetes are serious in nature and much research is being performed on different strategies to prevent and/or alleviate their symptoms. Numerous physiological mechanisms within the body relate to diabetes and obesity. Therefore, it is difficult to identify the exact molecules and reactions to target when hypothesizing the most efficient and effective prevention strategy. Of interest to this current research were the mechanisms of enzymatic digestion of lipids and carbohydrates, as well as the secretion of insulin-related hormones, which are discussed in subsequent sections.

Carbohydrate Digestion. Carbohydrates are molecules comprised of either one or many sugar units, including the monosaccharide glucose, and can be present in highly polymerized structures such as starches. Carbohydrates enter the bloodstream by being broken down into these monosaccharides in order to be more easily absorbed. Once ingested carbohydrates reach the small intestine, the pancreas secretes the enzyme α-amylase, which is responsible for hydrolyzing internal α-(1,4) glycosidic bonds within the carbohydrate molecule, breaking the larger compound down into smaller saccharide subunits.[103] **Figure 2.9** shows a basic representative mechanism by which α-amylase breaks a polysaccharide down into smaller substituents. This is the same general reaction by which the enzyme breaks down even larger compounds, such as starch, into smaller sugars so that they are more easily digested. Another carbohydrate-digesting enzyme of concern in this research is α-glucosidase. This enzyme is located on the brush-border of the small intestine and initiates the final step of carbohydrate

digestion, breaking α-(1,4) bonds to release glucose molecules.[15, 104] Figure **2.10** demonstrates a basic reaction of α-glucosidase with a polysaccharide, releasing terminal glucose units.

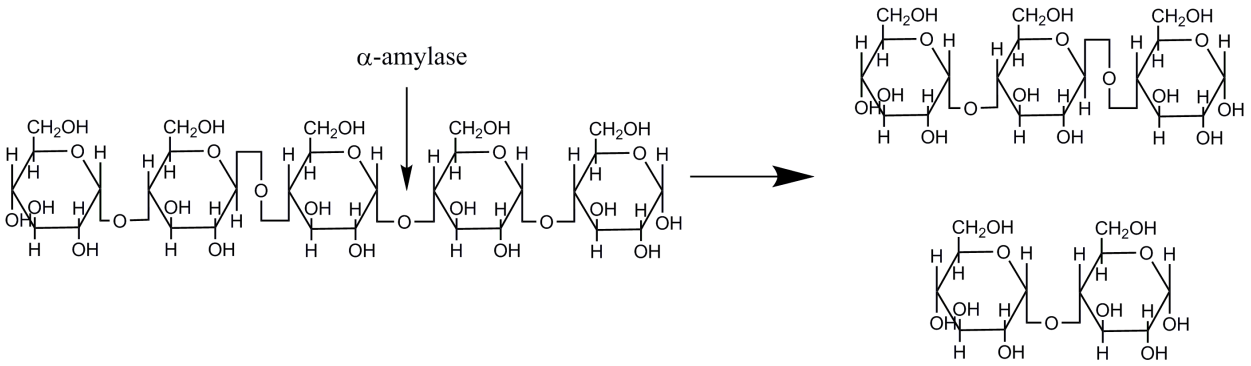


Figure 2.9. *Example mechanism by which α-amylase breaks a carbohydrate down into smaller saccharide units*

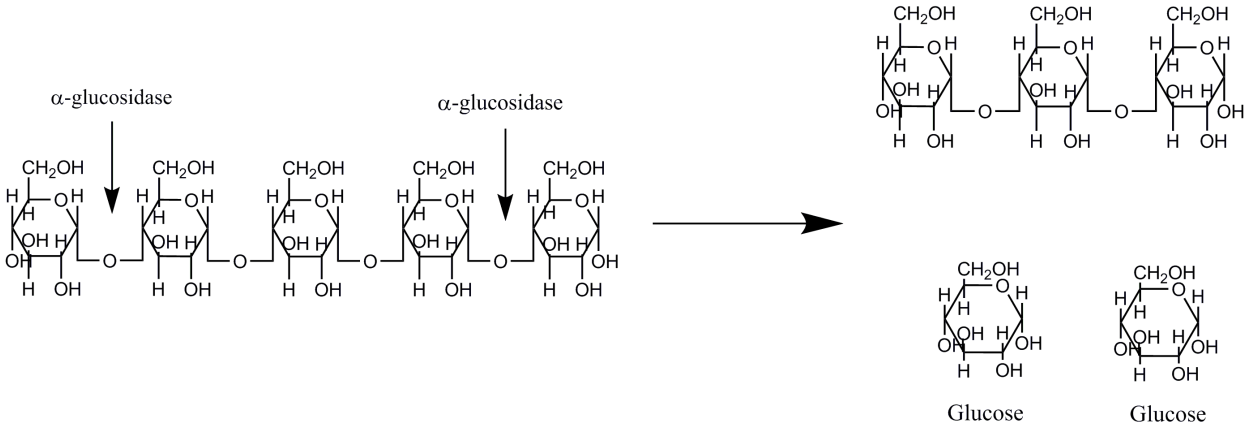


Figure 2.10. *Example mechanism by which α-glucosidase breaks down carbohydrates to release glucose molecules*

Glucose is rapidly absorbed after ingestion of carbohydrates because it is a major source of energy for the body.[105] However, rate and concentration of glucose absorbed is of major concern in the case of type-2 diabetes. If insulin is not adequately secreted, blood glucose levels remain high and the body is unable to regulate it properly. While there are drugs available on the

market to help treat these effects of diabetes, side effects and drug resistance are of major concern.[63] Therefore, researchers are focusing in on identifying natural substances that may alleviate some of these effects. Previous studies have shown that polyphenol-rich foods such as apple juice, berries, and red wine can reduce postprandial blood glucose levels in human subjects.[106-108] Results such as these also show promise for cocoa to have similar benefits related to carbohydrate digestion and regulation of glucose absorption into the blood.

Fat Digestion. Digestion of fats begins with chewing in the mouth and interactions with gastric juices in the stomach, which produce emulsion particles that act as substrates for lipases, the enzymes that break down triglycerides into free fatty acids and monoacylglycerols.[109] Fat cannot be absorbed by the small intestine without first being broken down by pancreas-secreted lipase.[18] Once the fat molecules are reduced to free fatty acids and monoglycerides, they are more readily absorbed in the small intestine and are transported to the bloodstream for use or storage. **Figure 2.11** demonstrates the general reaction by which lipase enzymes act to break down triglycerides into free fatty acids and monoglycerides.

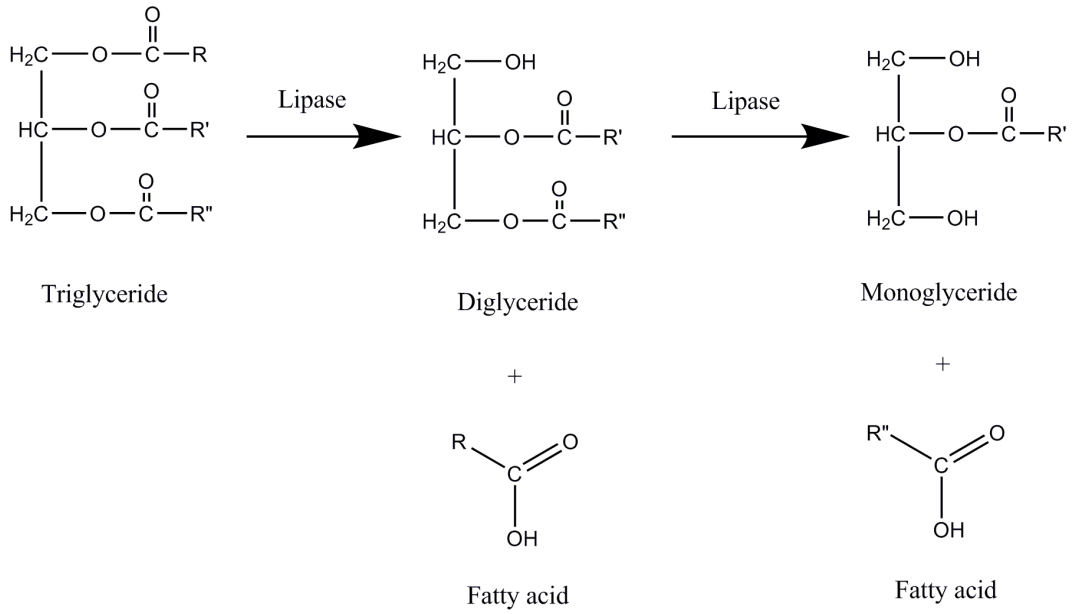


Figure 2.11. *Example reaction by which lipase breaks triglycerides down into free fatty acids and monoglycerides*

High levels of triglycerides in the body can be of major concern to overall health, leading to conditions such as obesity and other cardiovascular-related issues. Many natural remedies, including polyphenolic compounds and polyphenol-rich foods, have been assessed for their abilities to combat or reduce deleterious effects related to high levels of fat accumulation in the body. One study by Gutiérrez-Salmeán *et al*[105] observed the effects of (–)-epicatechin supplementation on lipid metabolism in normal and overweight human subjects. They observed drastic effects after just one dose of the compound including decreased respiration quotients (indicating increased fat oxidation) and alleviation of increases in glycaemia and triglyceridemia.[105] Although the beneficial effects were more noticeable in the overweight subjects, the researchers ultimately concluded that (–)-epicatechin has an acute effect on the modulation of lipid metabolism in relation to detrimental health conditions. Another study by Ali *et al*.[110] fed rats a high-fat diet supplemented with cocoa polyphenols and observed the effects on lipid metabolism. After four weeks of supplementation, those rats fed the high-fat diet + cocoa polyphenols demonstrated lower lipid levels in the liver and alleviation of increases in body weight and visceral fat accumulation. In another study, Rabadan-Chávez *et al*.[111] administered supplements of cocoa powder, cocoa extract, epicatechin, catechin, and procyanidin B2 to diet-induced obese rats. After eight weeks, the cocoa powder, cocoa extract, and epicatechin treatment groups demonstrated decreases in weight gain, fat mass, insulin resistance, lipogenesis, inflammation, and TNF-α levels, as well as increases in adiponectin in white adipose tissue, which helps regulate glucose levels and fatty acid breakdown.[111]

While there are many biomarkers that can be measured and numerous ways by which these flavanol compounds can act, the specific mechanisms still remain somewhat unknown. However, it is clearly demonstrated in these studies that flavanols, including those found in cocoa, do have an effect on lipid metabolism *in vivo* and have the potential to alleviate some of the adverse side effects related to extreme fat accumulation.

Incretins and Dipeptidyl Peptidase-IV. Upon ingestion of food, hormones known as incretins are secreted, which in turn, stimulate the pancreas to produce insulin in order to regulate blood glucose levels.[112] Two main incretins studied today include glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotrophic polypeptide (GIP). Levels of these hormones are generally low during a fasted state and increase quickly once food has been ingested, classifying

themselves as "nutrient-stimulated" hormones.[113, 114] GLP-1 is secreted by L cells in the distal intestine and suppresses glucagon to maintain normal blood glucose levels.[113] GLP-1 can also stimulate insulin secretion, help to slow gastric emptying, and increase satiety, all of which help to promote proper digestion and absorption of glucose and other nutrients.[115-117] GIP is secreted by K cells in the upper small intestine and also works to induce insulin release and maintain normal blood glucose levels.[113]

GLP-1 and GIP are substrates for the enzyme dipeptidyl peptidase-IV (DPP4), which is present on all cells and works to inactivate these incretins.[118] GLP-1 and GIP are rapidly degraded by DPP4, resulting in extremely short circulating half-lives (1-1.5 min for GLP-1 and about 7 min for GIP).[118-120] Once these hormones are inactivated, they can no longer remain active within the gastrointestinal tract, insulin is not properly secreted, and rises in blood glucose levels are not alleviated. Recently, tetrameric Cinnamtannin A2 (isolated and purified from cacao liquor) has shown to increase insulin and GLP-1 secretion *in vivo,*[121] which shows promise for cocoa procyanidins as an incretin-based therapy. Incretin-based therapies have been summarized to also have the ability to aid in dyslipidemia, hypertension, endothelial function, coronary artery disease, and heart failure.[122] Since GLP-1, GIP, and DPP4 all function based on blood glucose and insulin levels, they have great impact on the development and progression of diabetes. This is why the mechanism between DPP4 and these incretins are recently under examination, so that potential strategies to combat or reduce diabetes may be discovered.

Enzyme Inhibition. While there is much research being done with natural supplementation in order to reduce the effects of diabetes and obesity, our research was mainly concerned with enzyme-inhibition mechanisms. It is believed that by slowing the reaction of α-glucosidase, pancreatic α-amylase, pancreatic lipase, and DPP4 with carbohydrates, lipids, and incretins, we may observe effects such as reduced spikes in postprandial blood glucose levels, delayed or reduced fat accumulation, increased satiety, and proper function of glucose-related hormones. Because it is suggested that one of the main sites of activity for flavanols is in the lumen of the gastrointestinal tract, we are targeting digestive enzymes that are mainly secreted and available here as well (pancreatic α-amylase, pancreatic lipase, and α-glucosidase).[36, 51, 123] Additionally, since DPP4 activity has a strong influence on glucose homeostasis in relation to diabetes, we wanted to look further into its inhibition. Currently, there are pharmaceutical options available

on the market that act as inhibitors of these enzymes to alleviate effects of diabetes and fat accumulation. However, side effects of these drugs are of major concern, which is why there is an interest in the utilization of natural product remedies, which may not cause as severe side effects, if any.[63] The following four sections describe previous research that has been done related to the inhibition of α-amylase, α-glucosidase, lipase, and DPP4 and the drug products used on the market as inhibitors.

Inhibition of Pancreatic α-Amylase. Upon inhibition of α-amylase, larger carbohydrate molecules cannot be as easily or quickly broken down into smaller sugar molecules. We believe that if this is the case, glucose will not be absorbed into the blood as quickly and satiety could potentially be increased. A large body of research has been performed examining the effects of polyphenol extracts and/or polyphenol-rich foods on the inhibition of α-amylase. Products that have shown to inhibit α-amylase include: grape seed tannins[124], persimmon peel polymers[16], bean hulls with high concentrations of condensed tannins[125], tea polyphenols[126], strawberries and raspberries[127], and other plant extracts.[128, 129] A common finding in some of these studies was that inhibition of α-amylase increased as the degree of polymerization (DP) of the phenolic compounds increased,[16, 124, 127] indicating that larger polyphenols inhibited α-amylase more effectively than smaller compounds. It is commonly thought that enzyme inhibition depends on the DP of the compounds,[128] which was one hypothesis we attempted to address in this thesis work.

The drug used to inhibit α-amylase is called Acarbose. Acarbose is a mixed, non-competitive inhibitor of α-amylase, which has shown to reduce both fasting and postprandial blood glucose levels in humans.[103, 130] Treatment using Acarbose can reduce the risk of diabetes development by about 6% in impaired glucose tolerance patients over 3 years of administration, showing its promise as an anti-diabetes drug.[131] However, common side effects of Acarbose include gastrointestinal events and discomfort,[130] which is why researchers are searching for an alternative solution to slow carbohydrate digestion.

Inhibition of α-Glucosidase. Inhibition of α-glucosidase delays the breakdown of the α-(1,4) bonds in sugar molecules that release glucose, which is then absorbed into the blood. We believe that inhibition of this enzyme will delay glucose uptake and reduce dramatic spikes in

postprandial blood glucose. Acarbose is also used as a pharmaceutical to inhibit α-glucosidase, reducing postprandial blood glucose levels and delaying glucose absorption.[132] Similar to its interactions with α-amylase, Acarbose works to slow carbohydrate digestion, but through a competitive, reversible inhibition mechanism with α-glucosidase.[133] While it has shown to be an effective inhibitor of α-amylase, Acarbose is a more extensive inhibitor of α-glucosidase.[134, 135] However, the side effects of Acarbose remain of concern when being used as an inhibitor of α-glucosidase.

Polyphenol-rich products that have shown significant inhibition of α-glucosidase include blueberries[127, 136], blackcurrant[127], grape seed, green tea, and white tea extracts[137], black tea and black tea pomace[138], persimmon peel oligomers[16], fruit shell extracts[139], pear peel[140], small grape steam leaf[15], Pycnogenol (pine bark)[141], and purified compounds such as flavones[142] and luteolin.[135] Just as α-amylase inhibition is thought to depend on the presence of high DP compounds, it is thought that inhibition of α-glucosidase is due to low DP compounds.[136] This concept was demonstrated in some of these studies' results.[16, 127] However, other research found that inhibition increased with increasing DP[141]; therefore, it remains uncertain the exact reason as to why these polyphenolic compounds exhibit inhibition activities of α-glucosidase.

Inhibition of Pancreatic Lipase. Delaying the breakdown of triglycerides into free fatty acids and monoglycerides could potentially delay or reduce fat accumulation and weight gain if the body has more time to digest, convert, and store them properly. We also believe that the more time it takes for the lipids to be digested, this may send satiety signals to the brain, reducing intake of additional food. Orlistat is a drug used on the market to manage weight levels.[143] Orlistat inhibits lipid hydrolysis by pancreatic lipase and facilitates weight loss by inhibiting fat digestion and absorption by ~30%.[144] It has shown efficacy in multiple clinical trials, one showing body weight reduction of about 8.5% after 1 year of treatment.[145, 146] However, side effects of Orlistat may pose concern and use of natural products may be more ideal for consumers.

Polyphenol-rich products have also been studied as inhibitors of pancreatic lipase. Products that have shown inhibition of lipase include: tea polyphenols[126], green tea[147-149], white tea[148], grape seed extract[150], oligomeric apple procyanidins[151], raspberry, Arctic bramble, cloudberry, and strawberry.[17] Due to these results, a common conclusion made was that the

presence of galloyl moieties within the polyphenolic compounds, such as those commonly found in tea polyphenols, is significant in the inhibition of lipase.[18, 152] While this has not been proven to be a requirement for effective lipase inhibition, it does seem like it may be an important factor.

Inhibition of Dipeptidyl Peptidase-IV. Inhibition of DPP4 prevents the degradation of GLP-1 and GIP, allowing these hormones to have longer endurance in the gut, help to increase insulin levels, and maintain glucose homeostasis.[153, 154] Studies have shown that inhibition of DPP4 reduces glucose depletion by ~32%.[155] A large amount of the research related to DPP4 inhibition to date looks into inhibitory drugs rather than natural compounds or products. However, similar to Acarbose and Orlistat, side effects are of concerns utilizing DPP4 inhibitor drugs.[112]

Recent findings have demonstrated a few non-pharmaceutical products as inhibitors of DPP4, including: pepsin-treated whey proteins[156], grape seed procyanidin extract[153], and small-leaf grapes (*Vitis thunbergii* var. *taiwaniana*).[15] Another study recently found that anthocyanins from a blueberry-blackberry wine and compounds commonly found in citrus, berry, grape, and soybean, such as resveratrol, luteolin, apigenin, and flavone strongly inhibited DPP4 (all of which had lower IC_{50} values than the standard, Diprotin A).[157] Since not much research has been performed on the inhibition of DPP4 by natural products, we believed that further studies were warranted. It is largely unknown which types of compounds interfere with DPP4 and how they do so, but since polyphenols have proven to have health benefits in the past, there is promise for them as natural DPP4 inhibitors as well.

Cocoa as an Enzyme Inhibitor. As stated previously in this literature review, dietary supplementation with cocoa, cocoa extracts and/or flavanol-rich chocolate products may reduce blood pressure, hyperglycemia, insulin resistance and other symptoms of diabetes and obesity in animal models[8, 61-64] and human subjects.[65-69] One probable mechanism by which cocoa demonstrates these effects is through inhibition of digestive enzymes in the gut. Since cocoa beans are 12-18% polyphenols by dry weight and are known to share similarities in polyphenol content with natural products that have previously shown enzyme inhibition such as tea, berries, and grape seed extracts, they have the potential to be strong enzyme inhibitors as well.[7, 19, 53] To date, there is not much research on cocoa polyphenols as inhibitors of digestive enzymes except for that of Gu *et al*.[7], who compared the capabilities of unfermented cocoa liquor (*lavado*),

standard cocoa liquor, and Dutch-processed (alkalized) cocoa liquor to inhibit pancreatic α-amylase, pancreatic lipase, and phospholipase A_2. They determined that the liquors contained significantly different levels of polyphenols and that the *lavado* extract was the most effective inhibitor of all three enzymes.[7] These results are ultimately what prompted the research of this thesis, examining how levels of processing affect concentrations of cocoa polyphenols and their abilities to inhibit digestive enzymes. Recently, Kang *et al*[158] demonstrated the inhibition of α-glucosidase by cocoa extracts on total polyphenol and total proanthocyanidin bases. They found that cocoa extracts inhibited the enzyme in a dose-dependent manner and that inhibition was not dependent on proanthocyanidins, but potentially more on the presence of low molecular weight compounds. This was the first study (to our knowledge) examining the inhibition of α-glucosidase by cocoa polyphenols, which we believe a reason why our current research is important and necessary.

Additionally, inhibition of DPP4 by cocoa polyphenols has not yet been explored to the best of our knowledge. Since similarities are found between grape and cocoa procyanidins, our cocoa extracts may act in a similar way to inhibit DPP4 in order to ultimately maintain glucose homeostasis *in vivo*. Through the inhibition of all of these enzymes, we believe there to be great promise for cocoa flavanols to exhibit strong anti-diabetic and anti-obesity activities, which could be a major finding to progress the idea of non-pharmaceutical product remedies for these common, yet detrimental health conditions.

Protein Precipitation. Astringency, or the binding of salivary proteins, is a sensorial response that commonly takes place upon consumption of polyphenol-rich foods and beverages such as tea, wine, dark chocolate, and cider. It is thought that it is the polyphenols in wine that bind these salivary proteins and precipitate them in the mouth, thus inducing an astringent sensation[91]. It is thought that wine astringency increases with tannin concentration, degree of polymerization, and amount of galloyl subunits.[159, 160] It has been hypothesized that if higher molecular weight compounds are present, more energy is generated in their interactions with protein such as bovine serum albumin, thus precipitating the protein.[161] Because polyphenols can interact with proteins, they are at least partially responsible for the astringency sensation, formation of haze in beverages, and inhibition of enzymes.[27] Therefore, since enzymes are proteins by nature, we

hypothesized that there could be non-specific binding of the enzymes by cocoa polyphenols, thus contributing to inhibition.

Harbertson *et al*.[162] demonstrated the protein binding abilities of cocoa utilizing 50-2000 ppm solutions of cocoa monomers to octamers. They also found that precipitation increased with increased DP (monomers and dimers did not precipitate the BSA), which is a finding to explore further in relationship to cocoa polyphenols. Since it has been proven that these higher doses of cocoa polyphenols are able to precipitate protein, we believe that this ability could be correlated to the potential bioactivities of binding to and inhibiting digestive and DPP4 enzymes.

Oxidative Polymers and Polymeric Pigments. Pigments of fermented tea are often attributed to compounds known as oxidative polymers. Tea fermentation is generally an enzymatic process occurring when oxidative enzymes that are naturally present in tea, such as polyphenol oxidase and peroxidase, react with tea catechins, which are substrates for the enzymes.[163, 164] During this process, various reactions take place over time including the formation of theaflavins by the oxidation of B rings to quinones, decarboxylation, and addition of functional groups such as carbonyls.[164] Additionally, peroxidase oxidizes flavanols and polymerizes these theaflavins.[165] Theaflavins, thearubigins, and theabrownins are classes of these oxidative polymers and are the most general classifications of colored compounds found in tea.[166] During tea fermentation, monomeric flavanols are first oxidized to dimers, which are the theaflavin class of these compounds.[167, 168] Further oxidation, polymerization, and condensation of theaflavins with catechins and catechin derivatives first form thearubigins.[169] Continuation of these reactions result in highly polymerized theabrownin compounds, which may also incorporate other components such as proteins, lipids, and caffeine.[170, 171] Oxidative polymers not only differ in size and composition, but also in their solubilities and affinities for organic versus non-organic solvents. For example, it is well known that an organic solvent such as ethyl acetate is capable of extracting very small compounds, such as theaflavins. On the other hand, theabrownins are soluble in water, but not in ethyl acetate, butanol, and other organic solvents.[171] Therefore, it appears that as compound size increases, affinity for non-polar, organic solvents decreases. Previous studies have utilized tea to demonstrate some beneficial bioactivities that these compounds may possess. Honda and Hara[172] suggested anti-diabetic effects of tea theaflavins via an intestinal glucosidase mechanism in rats. Additionally, Gong *et al*.[173] demonstrated lipid-

lowering effects of tea theabrownins also in a rat model. These studies show promise for oxidative polymers to have beneficial effects *in vivo*.

We believe that there may be similar compounds formed during cocoa fermentation, contributing to the brown color of fermented cocoa and potentially some of cocoa's bioactivities. While tea fermentation is primarily enzymatic, cocoa fermentation is both enzymatic and microbial, incorporating yeasts, lactic acid bacteria, and acetic acid bacteria.[73] During cocoa fermentation, (–)-epicatechin undergoes oxidation and polymerization to form large, insoluble compounds; this reaction can either be non-enzymatic or enzyme-catalyzed by polyphenol oxidase.[12, 53] Also during fermentation, increases in terminal amino groups on cocoa polyphenols have been observed as well as the formation of additional amino acids, which then become precursors for browning reactions.[13, 174] Polymerization and incorporation of other compounds increases the amount of functional groups on the polyphenols, which can become highly reactive in further stages of processing. For example, during the aerobic stages of drying, enzymatic or spontaneous oxidation of (–)-EC can occur and lead to formation of melanin and melanoproteins, resulting in a brown color.[53] Also, monomers are oxidized to quinones and condensation of polyphenols with proteins occurs to form some of these large, complex structures.[175]

Additional processing such as roasting of cocoa involves further reactions that aid in the formation of complex structures. Due to high levels of oxygen, polyphenols can further bind with polymeric structures, thus increasing their brown color.[41] The amino acids formed during fermentation become precursors for Maillard browning during roasting, which further enhances the brown color and develops flavor compounds.[174] Maillard browning is a non-enzymatic process and involves condensation between carbonyl compounds (such as reducing sugars, aldehydes, ketones, and amino groups).[174] Amino acids and sugars together with polyphenols are highly reactive and are affected by pH conditions such as those in cocoa fermentation.[174] These are few among many types of reactions that can occur during cocoa processing. However, it is evident that there is much opportunity for numerous complex, highly-polymerized compounds to be formed.

Although the cocoa and tea fermentation processes are very different, they are conceptually similar, forming different compounds while affecting overall product taste, quality, appearance, etc. Therefore, we may be able to roughly correlate presence of compounds that act in a similar way as theaflavins, thearubigins, and theabrownins (extraction based on polarity) to

those formed in cocoa fermentation. Presence of these (or similar) compounds can be measured based on pigment and absorbance. We will refer to these compounds that contribute to color of the product as polymeric pigments, which can roughly be related to presence of oxidative polymers. Oxidative polymers in relation to cocoa has not yet been explored extensively. We believe that delving deeper into this new area will open the door to a potentially promising area of cocoa bioactivity, shifting the focus away from solely studying bioactivities of flavanol compounds.

Identification of these highly polymerized compounds is extremely difficult due to analytical limitations. First, separation and identification of these compounds has not yet been explored to a full extent. Since these compounds are so highly-polymerized and can incorporate a plethora of other constituents, analytical standards do not exist for all of the possible compound combinations. Additionally, specific analytical techniques used today are not yet capable of efficiently separating these large compounds, making it difficult to truly understand their structures. While this area has not yet been explored in depth, this provides many opportunities for scientists to begin preliminary studies on how to effectively separate, quantify, and identify these compounds. As this thesis work evolved over time, we became more interested in the bioactivities of these oxidative polymers that may be present in cocoa, shifting the focus away from cocoa flavanols as the only bioactive components and potentially providing a platform for a new area of research.

Conclusions. The exploration of the anti-diabetic and anti-obesity bioactivities of cocoa is a relatively new research area over the past few decades, therefore, there remains many unknowns that require further examination. While our current research delves into the enzyme-inhibiting activities of cocoa polyphenols, the mechanisms by which this occurs are still unclear. There has been previous research on enzyme kinetics that take place during these activities, however, we do not know if these are the only mechanisms that may occur in order to delay enzyme activity. Additionally, it is very well known that the fermentation and roasting steps of cocoa processing (among others) significantly decrease the levels of naturally occurring polyphenols in the beans. However, it is still widely unknown how exactly these changes in polyphenol levels and profiles affect overall bioactivities of cocoa related to diabetes and obesity. One would hypothesize that bioactivity increases with polyphenol concentration, however, there has been little research to

either prove or disprove this theory. The ultimate goal from a food processing standpoint would be to discover a way to process cocoa beans so that bioactivity is optimized (whether that means preserving levels of polyphenols or not) while also maintaining normal sensory attributes that chocolate consumers have grown accustomed to. Since polyphenols impart various challenges in the sensorial area, this would be a very difficult task to accomplish. This is why our research strove to determine if cocoa that has been processed and had decreased levels of polyphenols could still potentially be health-beneficial.

The most pressing question that arose while performing our research is if there are other classes of polyphenols that could be formed during cocoa processing that also contribute to its bioactivities. Most of the previous research on cocoa bioactivity has been focused on flavanol monomers, oligomers, and polymers. However, we believe it is possible that larger, more complex compounds could be formed during fermentation and roasting that could possess bioactivities as well. What is unknown with this question is how to effectively and specifically measure, quantify, and identify these compounds so we can know what compounds are being formed. We believe that if there is another classification of compounds that could provide such beneficial bioactivities, then it is important to begin identifying them in order to open up a whole new area of polyphenol chemistry research.

Our current studies aimed to address some of these unknowns and important questions that remain unanswered in this area of cocoa polyphenol bioactivity. Through the classification of polyphenol contents and measurements of enzyme inhibition *in vitro*, we strove to draw conclusions on how these changes are correlated to bioactivity. Additionally, we performed preliminary work on the measurement and quantification of this class of oxidative polymers in cocoa and correlated those with enzyme inhibition as well. This thesis work has not only reinforced and drawn new conclusions on work that has been done previously, but also provides a bridge to a new area of cocoa research that could have promise in identifying its health-benefits.

Chapter 3:

Loss of Native Flavanols during Fermentation and Roasting Does Not Necessarily Reduce Digestive Enzyme-Inhibiting Bioactivities of Cocoa*

Introduction

Consumption of flavanol-rich foods is thought to play a role in improving overall health and preventing the onset of certain chronic diseases.[19] On a per-weight basis, cocoa has the highest concentration of flavanols compared to any other food.[9, 10] The flavanols found in cocoa include monomeric flavanols (also known as flavan-3-ols), such as (–)-epicatechin (EC), (+)-catechin (+C), and (–)-catechin (–C), as well as procyanidins (PCs), which are flavanol oligomers and polymers (**Figure 1**).[56]

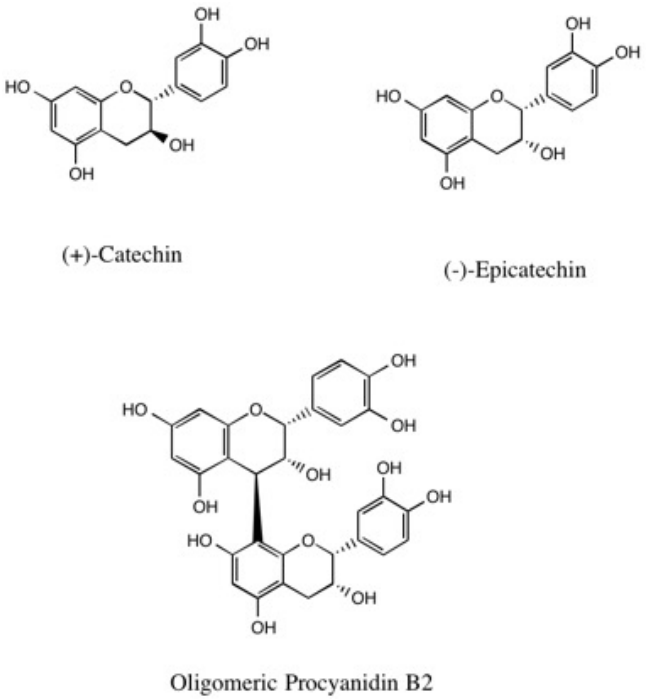


Figure 3.1 *Representative structures of monomeric and oligomeric flavanols*.

*The contents of this chapter are currently under revision for publication in the Journal of Agricultural and Food Chemistry.

Over 30% of the United States population is obese, which may lead to the development of cardiovascular disease, diabetes, cancer, and arthritis.[2] It is expected that 366 million people in the world will have diabetes by 2030, a 114% increase from 2000.[4] Cocoa has recently been explored as a potential complementary strategy to prevent and/or ameliorate diabetes. Dietary supplementation with cocoa, cocoa extracts and/or flavanol-rich chocolate products have been reported to reduce blood pressure, hyperglycemia, insulin resistance and other symptoms of diabetes and obesity in animal models[8, 63] and human subjects.[67, 68] These effects are thought to be primarily attributable to flavanols. Due to the relatively poor bioavailability of cocoa flavanols (particularly PCs larger than trimers),[51] one probable mechanism by which cocoa exerts these effects *in vivo* is inhibition of digestive enzymes in the gut.[7] Flavanol interaction with luminal digestive enzymes does not require absorption, nor is it reduced by Phase I-II metabolism or apical efflux transporters, which limit flavanol systemic bioavailability.[95, 97]

Carbohydrate-digesting enzymes include pancreatic α-amylase, which hydrolyzes α-(1,4) glycosidic bonds in starch and other carbohydrates, and α-glucosidase, which is located on the brush-border of the small intestine and further breaks down glycosidic linkages of carbohydrates to release glucose molecules.[103] Delaying or inhibiting carbohydrate digestion could reduce postprandial blood glucose absorption and maintain satiety. Pancreatic lipase hydrolyzes triglycerides into glycerol and free fatty acids.[109] Inhibition of pancreatic lipase could reduce lipid absorption and increase satiety, preventing fat accumulation and weight gain. Previous research has shown that polyphenols in tea, berries, and grape seed extracts have the ability to inhibit one or more of these three enzymes *in vitro* and/or *in vivo*.[17, 137, 176] Various drugs are available to inhibit these enzymes. Acarbose interferes with carbohydrate-digesting enzymes, thus delaying postprandial blood glucose levels and absorption; this can reduce the risk of diabetes development by about 6% in impaired glucose tolerance patients over 3 years of administration.[131] Orlistat inhibits pancreatic lipase, thus delaying fat digestion and absorption by ~30%[144] and has shown body weight reduction of about 8.5% after 1 year of treatment.[146] However, due to the undesirable side effects of these drugs, there is interest in dietary phytochemicals with similar activities as non-pharmaceutical alternatives.[63] Since cocoa beans are 12-18% polyphenols by dry weight and are known to share similarities in polyphenol content to tea, berries, and grape seed extracts, they have the potential to be strong enzyme inhibitors.[53]

Upon harvest, cocoa beans are typically heaped into piles and fermented by environmental microbiota. This process forms flavor precursors that further develop during the subsequent roasting step, producing characteristic cocoa flavors and defining quality.[12] Fermentation and roasting significantly decrease the polyphenol content of cocoa due to the high temperature conditions, oxidation, and changes in pH.[12, 53, 177] It is largely unknown how these changes in polyphenol composition affect the biological activities of cocoa, such as digestive enzyme inhibition, but it is generally thought that preservation of polyphenols is important for retaining bioactivity.[177] The impact of processing on cocoa bioactivity is beginning to be studied more closely. Gu *et al*.[7] found that unfermented cocoa liquor (*lavado*) extract was the strongest inhibitor of pancreatic α-amylase, pancreatic lipase, and phospholipase A_2 compared to standard and Dutch-processed (alkalized) cocoa liquors. Recently, Kang *et al*.[158] observed effective inhibition of α-glucosidase by cocoa extracts in a dose-dependent manner.

Our objective was to determine how fermentation and roasting impact the bioactivities of cocoa, and correlate bioactivity with chemical composition. We hypothesized that fermentation and roasting significantly would decrease the ability of cocoa to inhibit digestive enzymes, and that this decrease would be correlated with decreases in cocoa polyphenols.

Materials and Methods

Chemicals and Standards. Folin-Ciocalteu reagent, sodium carbonate, (+)-catechin hydrate (catechin • xH_20), 4-dimethylaminocinnamaldehyde (DMAC), potassium chloride, sodium acetate, benzyl mercaptan (use of this reagent must be performed under a fume hood with proper protective equipment due to its flammability, toxicity, and other safety hazards), pancreatic lipase (porcine pancreas), Trizma-HCl, Triton X-100, 4-nitrophenyl dodecanoate, Orlistat, α-glucosidase (*Sacchromyces cervesae*), *p*-nitrophenyl-α-D-glucopyranoside (nPNG), Acarbose, α-amylase (porcine pancreas), and red starch were obtained from Sigma (St. Louis, MO). Procyanidin B2 was obtained from Chromadex (Irvine, CA). Solvents were ACS grade or higher.

Cocoa Samples. Cocoa products representing various levels of processing were obtained. Unfermented cocoa beans (UB) and fermented cocoa beans (FB) were a gift from Cargill, Inc. (Minnetonka, MN). The beans were harvested in Malaysia and those that were fermented had undergone approximately four days of fermentation. **Table 3.1** provides all identifying information provided to us by Cargill, Inc. Unfermented (*lavado*) cocoa liquor (UL) and standard fermented cocoa liquor (FL) were a gift from The Hershey Co. (Hershey, PA). "Liquor" refers to the solid mass containing both cocoa solids and cocoa butter after roasting and grinding of the beans. Additional analyses were run by The Hershey Co. providing caffeine, theobromine, fat, and total polyphenol contents in the original liquor samples (**Table 3.2**).

Table 3.1 *Identifying information for cocoa beans provided by Cargill, Inc.*

Product name	Unfermented cocoa – Rainman	Fermented cocoa – Rainman
Date of Packaging/Manufacture	Oct, 2013	
City of Manufacture	Wormer, Netherlands	
Location of Harvest	Malaysia	
Lot Code/ID#	IVC13	
Shelf Life/Expiration Date	Oct, 2015	
Preparer's Name	Sonia Han	
Number of Days Fermented	N/A	4 d

Table 3.2 *Analysis of original cocoa liquors by The Hershey Co.*

Sample	Unfermented Liquor	Fermented Liquor
Caffeine (%)	0.15 ± 0.0153	0.13 ± 0.0153
Theobromine (%)	1.32 ± 0.144	1.16 ± 0.137
Fat (%)	53.8 ± 0.265	56.7 ± 0.94
Total Polyphenols on a Whole Product Basis (mg/g)	60.5 ± 3.5	29.5 ± 1.5

Cocoa Polyphenol Extraction. Approximately 110 g of each sample was frozen in liquid N_2 and then blended in a Waring laboratory blender on high speed for 1 min until ground into a powder. The powder was defatted with 400 mL hexane. The hexane/cocoa suspension was sonicated for 10 min, stirred for 5 min, and centrifuged for 5 min (5000 x *g*, 20°C). The supernatant was discarded and the defatting procedure was repeated. Residual hexane was evaporated at room temperature. Polyphenol extraction was then performed as follows: 400 mL of a 70:28:2 acetone:MilliQ water:acetic acid solution was combined with the dried, defatted cocoa material. The solution was sonicated for 10 min, stirred for 5 min, and centrifuged for 5 min. The supernatant was collected, and the extraction procedure repeated three more times and the supernatant extracts pooled until the supernatant was essentially colorless. Pooled supernatants were evaporated under vacuum on a rotary evaporator at 40°C until all the acetone had evaporated. The remaining cocoa extract (CE) was frozen at –80°C for 20 min, then freeze-dried for ≥ 2 d. Once dried, yield was recorded and the extract stored at –80°C. Additional analyses were run by The Hershey Co. providing caffeine and theobromine contents in the liquor extracts (**Table 3.3**).

Table 3.3 *Analyses of liquor extracts by The Hershey Co.*

Sample	Unfermented Liquor Extract	Fermented Liquor Extract
Caffeine (%)	0.995 ± 0.055	0.81 ± 0.03
Theobromine (%)	8.44 ± 0.385	7.96 ± 0.33

Determination of Total Polyphenol, Total Flavanol, and Total Anthocyanin Content.

The Folin-Ciocalteu colorimetric method was performed based on the method described by Dorenkott et al.[8] to determine the total polyphenol content in each of the cocoa samples. Cocoa extracts (*n*=4) were diluted with 40% EtOH to a final concentration of 0.2 mg/mL. 100 μL of each solution was mixed with 900 μL distilled deionized water and 2.5 mL 0.2 N Folin-Ciocalteu reagent. 2 mL sodium carbonate solution (7.5% w/v) was added to the samples and vortexed. After 2 h incubation at room temperature, the absorbance of the final solutions was read at 765 nm. Samples were compared to (+)-catechin hydrate and gallic acid standard curves performed in duplicate. Total polyphenol concentrations were expressed in units of mg catechin (+C) equivalents/g CE and mg Gallic Acid Equivalents (GAE)/g CE. Two standard curves were employed in order to report values as GAE (standard value reported in the literature) as well as catechin equivalents, which are the main monomeric flavanols in cocoa. On average, the Folin response expressed as C equivalents was ~84% of the response expressed as GA equivalents. This may be due to differences in catechin response, as well as the unknown catechin:water ratio in the (+)-catechin hydrate (catechin • xH_20) standard.

The 4-dimethylaminocinnamaldehyde (DMAC) colorimetric assay was performed as described by Payne et al.[46] with modifications to assess the total amount of flavanols in the cocoa extracts. The DMAC solution was prepared as follows: 3.0 mL stock HCl and 27 mL EtOH were combined, the solution was cooled for 15 min at 4°C, and 0.03 g DMAC was added and mixed thoroughly. Cocoa extracts (*n*=4) were diluted with EtOH to 100 ppm. Procyanidin B2 was diluted with 1:1 EtOH:water to final concentrations of 1, 10, 50, and 100 ppm in order to prepare a standard curve (*n*=2). In a 96 well plate, 50 μL of either EtOH blank, procyanidin B2 standard solution (1, 10, 50, or 100 ppm), or diluted cocoa extract (100 ppm) was added to individual wells. 250 μL DMAC solution was pipetted into each well and absorbance was read at 640 nm.

Concentrations of total monomeric anthocyanins were determined based on their color-changing nature in response to pH using the method of Lee et al.[39] with modifications. CE (120 mg cocoa extract, *n*=4) was dissolved in 1 mL dimethylsulfoxide (DMSO). In separate volumetric flasks, 100 μL of each diluted CE was further diluted to 5 mL with 0.025 M potassium chloride buffer (pH 1.0) and 0.4 M sodium acetate buffer (pH 4.5) (it was previously determined that the

maximum volume of DMSO added to the buffers without altering its pH was ~2% of the total volume, hence a 50x dilution was used for CE in DMSO). 200 μL of each solution was then transferred to a 96-well plate and read at 520 and 700 nm. When reading the plates, the wavelengths were pathlength corrected at 900/977 nm so that each absorbance value was normalized to a standard 1 cm pathlength. The total anthocyanin pigment concentration of each diluted sample, expressed as mg/L cyanidin-3-glucoside equivalents, was calculated as described by Lee et al.[39]. Further calculations were performed to determine anthocyanin concentration per gram cocoa extract. It is important to note that some negative values were obtained for the anthocyanin assay of FB and FL samples; any negative results obtained were automatically assumed to be zero values for calculation purposes.

Normal-phase HPLC Analysis. Normal phase HPLC was utilized as a qualitative measurement of the degree of polymerization (DP) profile of the flavanols present in the samples as described by Dorenkott *et al*.[8] CE samples were diluted to a final concentration of 20 mg/mL with 70:28:2 acetone:water:acetic acid and filtered into HPLC vials using Microsolv syringe filters (13 mm diameter, 0.22 μm nylon membrane with propylene housing). Samples were analyzed on an Agilent Technologies (Santa Clara, CA) 1260 Infinity HPLC equipped with a solvent degasser, quaternary pump, autosampler with temperature control, thermostat column compartment, and a fluorescence detector (FLD). The column used for separation was a Develosil Diol Column (100Å, 250 x 4.6 mm, 5 μm particle size) at 35°C with a Luna HILIC guard column (4 x 3.0 mm ID SecurityGuard cartridge and cartridge holder) (Phenomenex, Torrance, CA). Binary gradient elution was performed at a flow rate of 1 mL/min with Phase A consisting of 2% acetic acid (v/v) in ACN and Phase B composed of 2% acetic acid (v/v) and 3% ddH_2O (v/v) in MeOH. The gradient was as follows: 93% A at 0 min, 93% A at 3 min, 62.4% A at 60 min, 0.0% A at 63 min, 0.0% A at 70 min, 93.0% A at 76 min, 7.0% B at 0 min, 7.0% B at 3 min, 37.6% B at 60 min, 100.0% B at 63 min, 100.0% B at 70 min, and 7.0% B at 76 min. FLD wavelength for excitation was 230 nm and 321 nm for emission. The samples were held at 5°C in the autosampler and 5 μL of each sample was injected into the HPLC. Two runs (n=2) were performed for each cocoa sample. It should be noted that uncalibrated fluorescence chromatograms are predominantly qualitative, as they do not accurately represent relative

quantitative flavanol composition between peaks (response factors for fluorescence detection decrease rapidly with increasing molecular weight for procyanidins[178]).

Thiolysis. Thiolysis was performed in order to determine the mean degree of polymerization (mDP) of flavanols in each cocoa sample based on the procedure of Dorenkott *et al.*[8] Cocoa extracts (n=4) were diluted with methanol to 0.5 mg/mL. 50 μL of these solutions, 50 μL HCl reagent (3.30% HCl in water), and 100 μL benzyl mercaptan reagent (5% benzyl mercaptan in methanol) were combined in microcentrifuge tubes and placed in a 90°C water bath for 5 min. The tubes were then cooled on ice for 5 min to stop the reaction. Unthiolyzed controls were prepared with 50 μL diluted cocoa extract solution and 150 μL methanol without heating. To prepare for LC/MS analysis, 100 μL of each sample was combined with 900 μL of a 95:5 0.1% formic acid in water (phase A):0.1% formic acid in ACN (phase B) solution in an HPLC vial. It is important to note that this reaction must be performed under a fume hood with proper personal protective equipment due to the flammability, toxicity, and other safety hazards of benzyl mercaptan. Figure 3.2 demonstrates the overall thiolysis reaction that occurs to release monomeric units and benzylthioether derivatives to measure mDP.

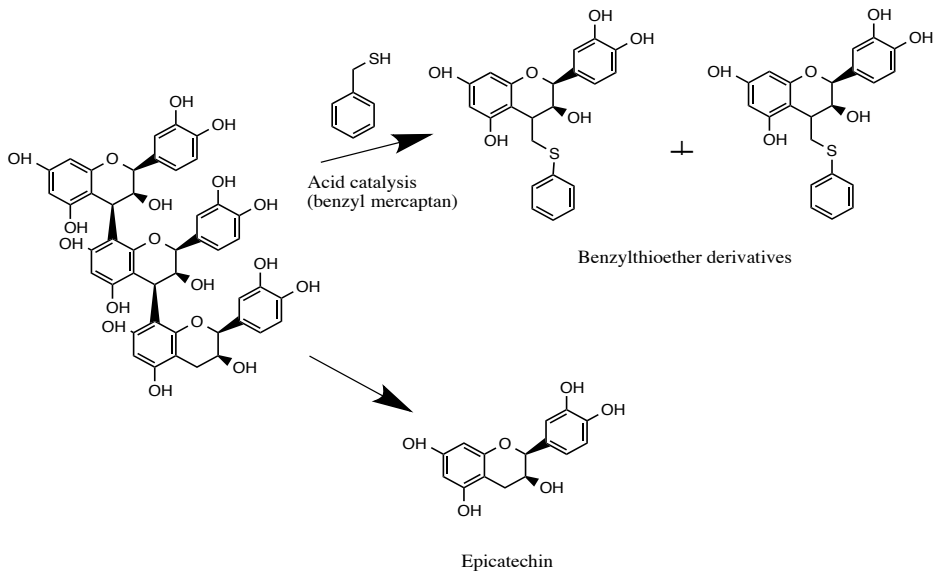


Figure 3.2 *Thiolysis reaction of a flavanol trimer*

20 μL of each solution was injected into the UPLC-MS/MS and the released flavanol units and benzylthioether derivatives were quantified. Samples were analyzed on a Waters Acquity H-class separations module with an Acquity UPLC HSS T3 column (2.1 mm x 100 mm, 1.8 μm particle size) at 40°C. Binary gradient elution was performed using 0.1% formic acid in water (Phase A) and 0.1% formic acid in acetonitrile (Phase B). Solvent flow rate was 0.6 mL/min and the linear gradient elution was the following: 95% A (0-0.5 min), 65% A (6.5 min), 20% A (7.5-8.6 min), 95% A (8.7–10.5 min). (–)-electrospray ionization (ESI) together with tandem mass spectrometry (MS/MS) was used to analyze UPLC effluent on a Waters Acquity triple quadrupole (TQD) MS. (–) mode electrospray ionization (ESI) was performed with capillary, cone, and extractor voltages of –4.24 kV, 30.0 V, and 3.0 V respectively. Source temperature was 150°C and desolvation temperature was 400°C. Cone gas flowed at a rate of 75 L/h and desolvation gas at a rate of 900 L/h. Argon (0.25 mL/min) was used as the collision gas in MS/MS. Multi-reaction monitoring (MRM) with a mass span of 0.2 Da was performed on parent ions and collision-induced dissociation (CID) on daughter ions. Inter channel delays and inter scan time was 1.0 s each. The MRM parameters for the detected compounds are shown in **Table 3.4**. By the nature of the thiolysis reaction, calculated mDP does not take into account the amount of native monomers originally present in the extracts. Therefore, an additional calculation was performed to account for these monomers and reported as DP of total flavanols. mDP of oligomers and polymers and mDP of total flavanols (accounting for native monomers) were calculated as follows:

$$mDP\ (O+P) = \frac{net\ number\ of\ monomers + net\ number\ of\ thiolytic\ derivatives}{net\ number\ of\ monomers}$$

$$mDP\ (total\ flavanols) = \frac{total\ monomers + net\ number\ of\ thiolytic\ derivatives}{total\ monomers}$$

Table 3.4 MRM Settings for the detection of monomeric flavanols and benzylthioether derivatives generated during thiolysis by UPLC-MS/MS.

Compound	Retention time (t_R) (min)	MW (g mol^{-1})	[M – H]$^-$ (m/z)	Daughter Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
C/EC	2.5 – 4.5	290.142	288.98	245.05	40	14
ECG	4.0 – 6.0	441.952	440.92	169.00	38	16
C/EC benzylthioether derivative	6.75 – 8.5	412.031	410.94	124.97	30	18
ECG benzylthioether derivative	7.0 – 8.25	563.824	563.05	287.06	38	16

Inhibition of α-Amylase. Inhibition of α-amylase was measured based on the method of Gu et al.[7] with modifications. α-Amylase from porcine pancreas was diluted with a 20 mM phosphate buffer (pH 6.9) containing 6.7 mM sodium chloride to a concentration of 3000 U/mL and centrifuged at 1,000 x *g* for 10 min. The supernatant was diluted 100-fold with buffer to a final concentration of 30 U/mL for the enzyme working solution. Cocoa extract samples were dissolved in DMSO and further diluted with distilled deionized water so that each sample tube contained only 10% DMSO. Red Starch solution (1 g Red Starch in 50 mL 0.5 M KCl) and the enzyme solution were pre-incubated in a 37°C water bath for 5 min. In a 96-well plate, 25 μL Red Starch solution, 20 μL distilled deionized water, 5 μL cocoa extract (0-2000 μg/mL in reaction; *n*=6), and 20 μL enzyme solution were combined. Plates were covered and incubated in the 37°C water bath for 20 min. To end the reaction, 200 μL 95% EtOH was added to each well. The plates were recovered, briefly placed on a plate shaker to mix contents, and centrifuged (6000 x g, 30 min, 4°C). 200 μL of the supernatants were transferred to a new 96-well plate and the absorbance was read at 510 nm. The 0 μg/mL sample was used as the negative control to measure full α-amylase activity. Acarbose was diluted to the same concentrations as the cocoa extracts using DMSO and distilled water and used as the positive control. The different concentrations of cocoa extract solutions were compared to the controls and expressed as % α-amylase activity. Activity was calculated as follows:

$$\%\,\alpha - Amylase\ Activity = \left(\frac{A_{I,S,E}}{\bar{A}_{S,E}}\right) x\ 100$$

Where:

$A_{I,S,E}$ = the individual absorbance value of the product of the inhibitor, substrate, and enzyme at each inhibitor dose

$\bar{A}_{S,E}$ = the average absorbance value of the reaction with substrate and enzyme without inhibitor (0 μg/mL)

Inhibition of α-Glucosidase. An assay described by Striegel et al.[138] was employed with modifications to examine inhibition of α-glucosidase by cocoa extracts. A 0.1 M phosphate buffer (pH=6.9) was used to prepare the following reagents: α-glucosidase solution (1.0 U/mL)

and a 1 mM *p*-nitrophenyl-α-D-glucopyranoside solution. Cocoa extracts were dissolved in DMSO and further diluted with distilled deionized water so that each sample tube contained only 10% DMSO. In a 96-well plate, 50 μL cocoa extract (0-2000 μg/mL in the reaction; *n*=6) and 100 μL of the α-glucosidase solution were combined and incubated at room temperature for 10 min. 50 μL of 1 mM *p*-nitrophenyl-α-D-glucopyranoside solution was added to each well and the absorbance was read at 405 nm. Samples were incubated at 25°C for 5 min and the absorbance was read at 405 nm again. The 0 μg/mL sample was used as the negative control to measure full α-glucosidase activity. Acarbose was diluted to the same concentrations as the cocoa extracts using DMSO and distilled water and used as the positive control. The different concentrations of cocoa extract solutions were compared to the controls and expressed as % α-glucosidase activity. Activity was calculated as follows:

$$\%\ \alpha - Glucosidase\ Activity = \left(\frac{\Delta A_{sample}}{\Delta \bar{A}_{blank}}\right) x\ 100$$

Where:

ΔA_{sample} = the change in the individual absorbance value of the product of the inhibitor, substrate, and enzyme at each inhibitor dose before and after incubation

$\Delta \bar{A}_{blank}$ = the average change in absorbance of the blank (0 µg/mL) before and after incubation

Inhibition of Pancreatic Lipase. An assay to determine inhibition of pancreatic lipase by cocoa extracts was performed as described by McDougall et al.[17] with modifications. Pancreatic lipase was diluted to 10 mg/mL in distilled deionized water, centrifuged at 17,000 x *g* for 5 min, and the supernatant was used as the working enzyme solution. To prepare the substrate solution, a 5 mM sodium acetate in 99:1 water:Triton X-100 solution (pH=5.0) was prepared, to which 4-nitrophenyl dodecanoate was added at 0.08% (w/v), heated in boiling water to help dissolve the fatty acid, then cooled to room temperature. Cocoa extracts were dissolved in dimethylsulfoxide (DMSO) and further diluted with distilled deionized water so that each sample tube contained only 10% DMSO. In a 96 well plate, 80 μL 100 mM Tris buffer (pH=8.2), 30 μL lipase solution (1.429 mg/mL in reaction), 10 μL diluted cocoa extract solution (0-2000 µg/mL in reaction, *n*=6), and 90 μL substrate solution (0.0343% in reaction) were combined. A second plate was

prepared replacing the enzyme solution with distilled deionized water in order to account for the pigments naturally present in the substrate and extracts. The plates were covered and incubated in a 37°C water bath for 2 h, oscillating at 40 rpm. After incubation, the plates were dried off and the absorbance was read at 400 nm. The 0 μg/mL sample was used as the negative control to measure full lipase activity. Orlistat was diluted to the same concentrations as the cocoa extracts using DMSO and distilled water and used as the positive control. The different concentrations of cocoa extract solutions were compared to the controls and expressed as % lipase activity. Activity was calculated as follows:

$$\%\ Lipase\ Activity = \frac{(A_{I,S,E} - \bar{A}_{I,S})}{(\bar{A}_{S,E} - \bar{A}_{S})}\ x\ 100$$

Where:

$A_{I,S,E}$ = the individual absorbance value of the product of the inhibitor, substrate, and enzyme at each inhibitor dose

$\bar{A}_{I,S}$ = the average absorbance value of the inhibitor and substrate (no enzyme) reaction

$\bar{A}_{S,E}$ = the average absorbance value of the reaction with substrate and enzyme without inhibitor (0 μg/mL)

$\bar{A}_{S}$ = the average absorbance value of the substrate without enzyme and inhibitor

Data Analysis and Statistics. Dixon's Q-test (α=0.05) was performed to detect and eliminate outliers if necessary. Origin and profile of the beans used to make liquors was unknown; therefore, we treated the four extract samples as independent treatment groups. We assumed that the liquors were produced from the same beans and that those beans were roasted under the same conditions for discussion purposes; the fact that the beans and liquors were not from the same source is a limitation of this study. Data for the Folin-Ciocalteu, DMAC, anthocyanins, and thiolysis assays were analyzed by one-way ANOVA; if a significant treatment effect was detected, Tukey's HSD *post hoc* test was performed to determine differences between treatment means. Median inhibitory concentration (IC_{50}) values for α-glucosidase and α-amylase were calculated using a four parameters sigmoidal analysis. Lipase data did not fit a sigmoidal curve, therefore linear regression was employed; furthermore, since less inhibition was observed for lipase, IC_{25} values were calculated as opposed to IC_{50} (IC_{25} represents the concentration at which 25% of enzyme activity

was inhibited). IC_{25} is also used in inhibition analysis[179] and thus was utilized as an arbitrary parameter which resulted in more accurate values instead of IC_{50} in the case of lipase. Enzyme inhibition values (%I) at individual concentrations were analyzed by one-way ANOVA; if a significant treatment effect was found, Tukey's HSD was then performed to determine differences between treatment means. Statistical significance was defined as $p < 0.05$. Analyses were performed on Prism v6.0f (GraphPad, La Jolla, CA).

Results

Cocoa Products and Extracts. Photographs of cocoa products and their CEs are shown in **Figure 3.3**. The purple color of UB and UL extracts suggests significant anthocyanin content. CE yields for UB, FB, UL, and FL were 98.7, 86.0, 96.6, and 94.0 mg/g starting product, respectively. Relative extract yields compared to the highest sample yield were 100%, 87.1%, 97.9%, and 95.2% for UB, FB, UL, and FL, respectively. While there were slight differences in extract yields (the largest difference between FB and UB), yields were similar enough that we compared results of equal extract concentrations for enzyme assays.

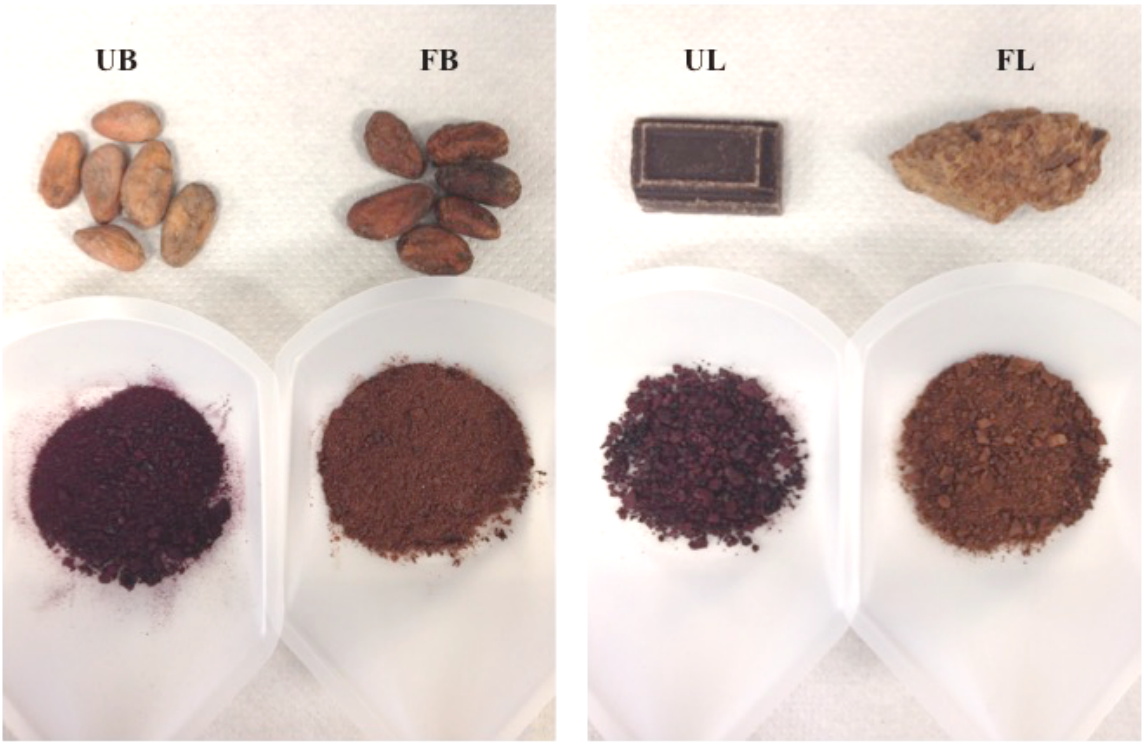


Figure 3.3 Original cocoa products and their corresponding extracts. From left to right: unfermented beans (UB), fermented beans (FB), unfermented liquor (UL), and fermented liquor (FL).

Polyphenol Characterization. Total polyphenol, flavanol, and anthocyanin contents of CEs are shown in **Figure 3.4 A-C**. Overall, there were significant decreases in all of these measurements between the unfermented and fermented products for both beans and liquors. Total polyphenol contents in the CE samples ranged from 171-390 mg C equivalents/g CE [232-432 mg gallic acid (GA) equivalents/g CE]. There was a 2.3-fold decrease in total polyphenols from UB to FB, and a 1.5-fold decrease from UL to FL. Based on statistical analysis, total polyphenol contents were UB = UL > FL > FB. For total flavanols, contents ranged from 64.7-327 mg PCB2 equivalents/g CE. There was a 4.8-fold decrease in total flavanols from UB to FB, and a 2.6-fold decrease from UL to FL. Statistical analysis indicated that flavanol contents were UB > UL > FB = FL. For total anthocyanins, contents ranged from 0-4.85 mg cyanidin-3-glucoside equivalents/g CE. There was a ~37-fold decrease in anthocyanins from UB to FB and a total loss from UL to FL. Based on statistical analysis, relative anthocyanin contents were UB > UL > FB = FL.

Normal Phase HPLC Analysis and Thiolysis. HPLC chromatograms of CEs are shown in **Figure 3.4E** as a qualitative profile of DP. Under these conditions, monomeric flavanols elute first, followed by elution of larger compounds, with retention time being proportional to molecular weight/DP.[8] UB had the largest peaks throughout the entire DP range, including a significant polymer "hump" (last eluting peak). UL also exhibited large peaks throughout the entire DP range, albeit smaller than those for UB. Overall, there was a significant decrease in concentration of flavanols, particularly monomers and oligomers, between unfermented and fermented samples for both beans and liquors. mDP values are shown in **Figure 3.4D**. A significant increase of mDP from UB to FB (2.85 ± 0.082 and 4.16 ± 0.077, respectively) was observed, whereas values for UL and FL were not different (2.51 ± 0.13 and 2.39 ± 0.041).

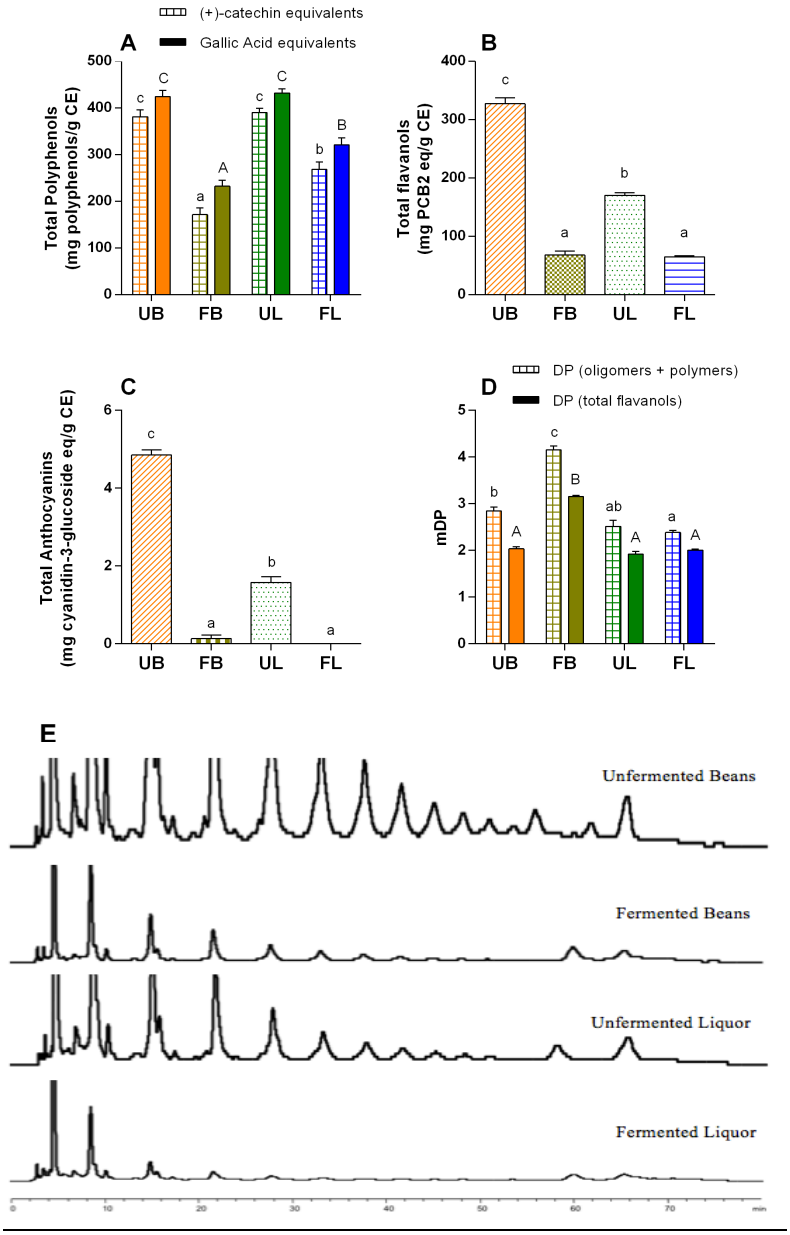


Figure 3.4 (A) Total polyphenols in each extract from the Folin-Ciocalteu assay expressed in catechin and gallic acid equivalents. (B) Total flavanols from the DMAC assay expressed in procyanidin B2 equivalents. (C) Total anthocyanins expressed in cyanidin-3-glucoside equivalents. Note: due to the nature of the anthocyanins calculation, any negative results obtained were made zero values. (D) Average flavanol degree of polymerization measured by thiolysis. DP (oligomers + polymers) represents the mDP measured from the released monomers and derivatives. For DP (total flavanols), native monomers in the CE were accounted for in the calculation.(E) Normal phase HPLC chromatograms for UB, FB, UL, and FL extracts expressed in fluorescence units (LU) over time (min). The scale for all four chromatograms is 100 LU.

Enzyme Inhibition. CE dose-dependently inhibited the activity of α-glucosidase, α-amylase, and pancreatic lipase (**Figure 3.5**). For α-glucosidase, UL, FL, Acarbose (+ control), FB, and UB had IC_{50} values of 90.0, 109, 158, 200, and 218 μg/mL, respectively **(Table 3.5)**. At 200 μg/mL, UL and FL were significantly better ($p<0.05$) inhibitors than Acarbose, whereas UB, FB, and Acarbose had similar activities. Cocoa extracts were not as potent as + controls in inhibiting α-amylase and pancreatic lipase. For α-amylase, Acarbose, FL, FB, UL, and UB had IC_{50} values of 54.3, 183, 404, 583, and 971 μg/mL, respectively (**Table 3.5)**. For lipase, IC_{25} values for Orlistat, UB, FB, UL, and FL were 26.3, 65.6, 107, 279, and 367 μg/mL, respectively (**Table 3.5)**. It should be noted that the lipase assay showed accuracy only up to 200 μg/mL concentrations, which is the data presented in **Figure 3.5**. Use of higher concentrations resulted in extreme variability, suggesting some form of interference with the assay. Since the cocoa extracts contain brown pigments, at such concentrated solutions (500-2000 μg/mL) this could be a potential source of interference with the assay. Full data sets demonstrating this variability can be found in **Figure 3.6**.

Table 3.5. *Enzyme inhibition parameters for cocoa extract samples and positive controls*

Enzyme	Parameter[a]		Inhibitor[c]				
			+ Control[b]	UB	FB	UL	FL
α-Glucosidase	IC_{50} (μg/mL)		157.9	217.8	199.5	89.98	108.6
	R^2		0.7145	0.793	0.9376	0.9899	0.8245
	% I	100 μg/mL	36.31 ± 0.924^{c}	19.17 ± 0.968^{d}	22.83 ± 0.753^{d}	55.09 ± 3.147^{a}	45.35 ± 2.854^{b}
		200 μg/mL	52.93 ± 1.460^{b}	47.86 ± 7.232^{b}	51.29 ± 1.500^{b}	86.31 ± 0.811^{a}	78.39 ± 2.237^{a}
α-Amylase	IC_{50} (μg/mL)		54.3	970.5	404.0	583.4	183.3
	R^2		0.9422	0.6624	0.9663	0.626	0.8566
	% I	200 μg/mL	65.79 ± 6.10^{a}	-1.14 ± 13.48^{b}	-1.67 ± 1.26^{b}	17.33 ± 10.86^{b}	22.97 ± 16.45^{ab}
		500 μg/mL	71.00 ± 2.65^{a}	3.61 ± 9.69^{c}	53.64 ± 8.10^{a}	15.05 ± 7.96^{bc}	38.41 ± 9.41^{ab}
		1000 μg/mL	76.42 ± 2.01^{a}	18.57 ± 9.42^{c}	60.53 ± 7.06^{ab}	32.07 ± 3.95^{c}	35.91 ± 8.74^{bc}
Pancreatic Lipase	IC_{25} (μg/mL)		26.3	65.6	107.2	279.4	366.7
	R^2		0.5741	0.7165	0.5183	0.7641	0.6916
	% I	200 μg/mL	62.68 ± 1.62^{a}	47.60 ± 5.71^{ab}	41.53 ± 9.069^{abc}	18.46 ± 3.09^{bc}	14.85 ± 7.23^{c}

[a]IC_{50}: median inhibitory concentration (concentration resulting in 50% inhibition compared to uninhibited activity), R^2: goodness of fit for the chosen model, %I: % inhibition of enzyme activity at the indicated concentration (due to the large range of concentrations tested, only concentrations close to the IC_{50} values were selected for presentation in the table), IC_{25}: concentration resulting in 25% inhibition compared to uninhibited activity

[b]Positive inhibitor controls for each assay: α-glucosidase and α-amylase (Acarbose) and pancreatic lipase (Orlistat)

[c]For % inhibition, values with different letter superscripts in the same row are significantly different (one-way ANOVA, Tukey's HSD post hoc test between all means, $p < 0.05$)

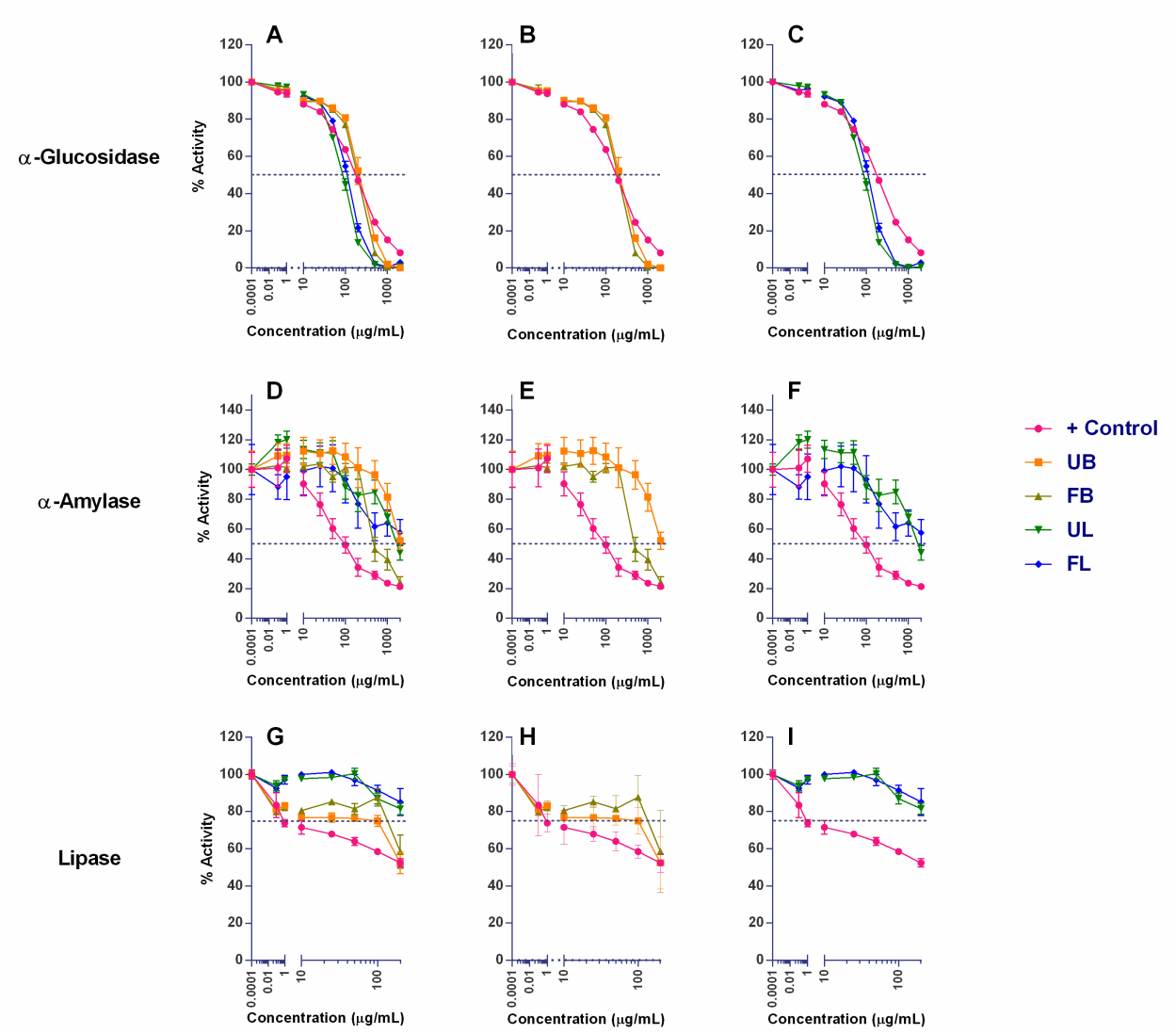


Figure 3.5 (A) α-Glucosidase % activity for all CE samples versus Acarbose control; (B) α-Glucosidase % activity for unfermented and fermented bean CEs versus Acarbose; (C) α-Glucosidase % activity for unfermented and fermented liquor CEs versus Acarbose; (D) α-Amylase % activity for all CE samples versus Acarbose control; (E) α-Amylase % activity for unfermented and fermented bean CEs versus Acarbose; (F) α-Amylase % activity for unfermented and fermented liquor CEs versus Acarbose; (F) Lipase % activity for all CE samples versus Orlistat control; (G) Lipase % activity for unfermented and fermented bean CEs versus Orlistat; (H) Lipase % activity for unfermented and fermented liquor CEs versus Orlistat. Dotted line represents 50% activity compared to uninhibited enzyme for α-glucosidase and α-amylase and 75% activity for lipase. Activity at 0 μg/mL was plotted as 0.0001 μg/mL in order to facilitate graphing on a logarithmic scale. Note: Graphical comparisons of UB to UL and FB to FL are not shown because bean and liquor samples were not from the same source.

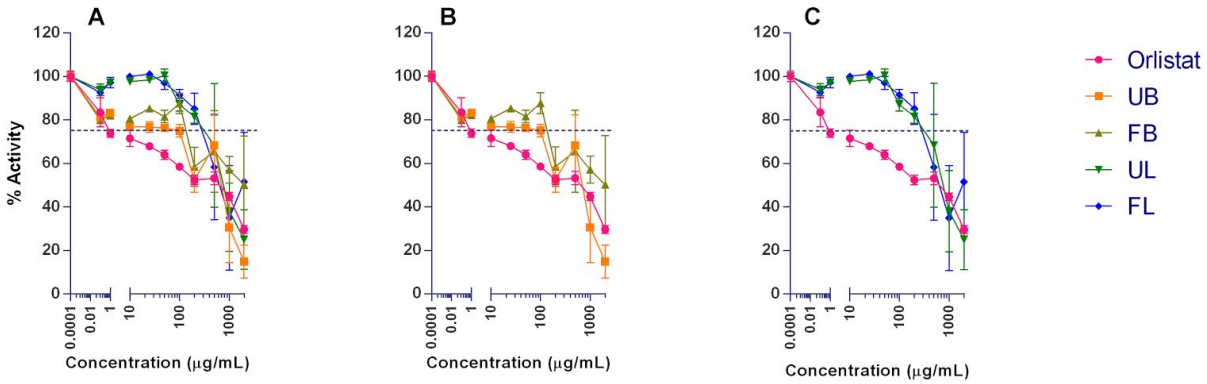


Figure 3.6. Pancreatic lipase % activity at all inhibitor concentrations for: (A) all CE samples versus Orlistat control; (B) unfermented and fermented bean CEs versus Orlistat; (C) unfermented and fermented liquor CEs versus Orlistat. Dotted line represents 75% activity compared to uninhibited enzyme. Activity at 0 μg/mL was plotted as 0.0001 μg/mL in order to be graphed on a logarithmic scale. Note: Graphical comparisons of UB to UL and FB to FL are not shown because bean and liquor samples were not from the same source.

Discussion

Significant losses of polyphenols and flavanols were seen between unfermented and fermented samples; however, this did not uniformly affect bioactivity of the extracts as predicted, suggesting that factors other than native polyphenol/flavanol contents contribute to digestive enzyme inhibition. Additionally, to the best of our knowledge, this is one of the first studies examining the impact of cocoa polyphenols on α-glucosidase activity. The data presented in this study suggest that polyphenolic extracts of unfermented and fermented cocoa beans and liquors are effective inhibitors of α-glucosidase and moderate inhibitors of pancreatic α-amylase and lipase.

A goal of this research was to objectively evaluate the relative health benefits of cocoa that has undergone fermentation and roasting processes, using representative samples. The IC_{50} and IC_{25} values in **Figure 3.7** facilitate examination of how fermentation and roasting affected enzyme inhibition abilities. Since beans and liquors were obtained from different sources, we cannot determine definite effects of roasting on enzyme inhibition (comparisons of UB to UL and FB to FL), but we use our data to suggest what changes may occur and to justify additional studies using the same raw material. IC_{50} (μg/mL) increased for α-glucosidase as follows: UL<FL<FB<UB, suggesting that roasting enhanced inhibition of α-glucosidase, as the liquors had the lowest IC_{50} values (**Figure 3.7A)**. However, there does not seem to be clear evidence of any effects of fermentation on inhibitory activity, which might be one of the most critical findings of this study. IC_{50} values for α-amylase were FL<FB<UL<UB suggesting that both fermentation and roasting improved cocoa's α-amylase inhibitory capabilities since fermented samples had the lowest IC_{50} values and between the two sample sets, roasted samples were lower than non-roasted (**Figure 3.7B**). This was a novel finding since our initial hypothesis (that processing effects on cocoa polyphenols would decrease inhibition effectiveness) was disproved by these data. Conversely, both fermentation and roasting attenuated inhibition of lipase as the IC_{25} values were UB<FB<UL<FL (**Figure 3.7C**). While it remains unclear the exact mechanism or compounds by which these differences occur, they might be attributed to changes in flavanol DP and composition. Although we have not studied further cocoa processes (alkalization, addition of milk, sugar, etc.), these initial steps of fermentation and roasting have proven to enhance potential health benefits in the cases of α-glucosidase and α-amylase inhibition.

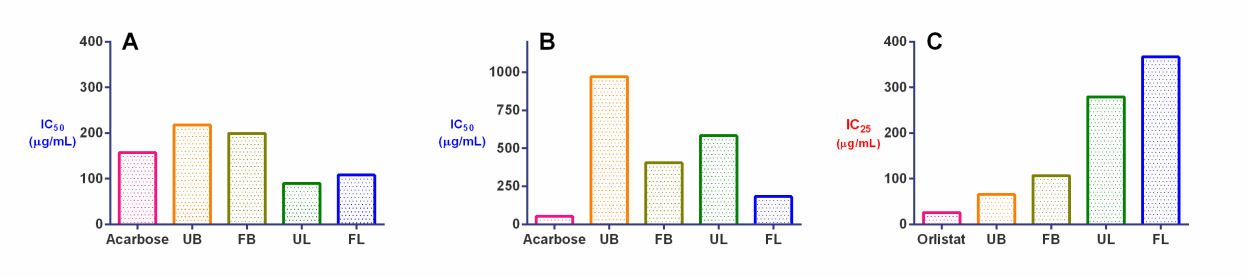


Figure 3.7. Comparison of IC values to visualize effects of fermentation and roasting on inhibition of (A) α-glucosidase; (B) α-amylase; and (C) pancreatic lipase.

In contrast with the bioactivity data, we observed 2.3- and 1.5-fold losses of total polyphenols between UB/FB and UL/FL, respectively (**Figure 3.4A**). These data correlate with the well-known phenomenon that fermentation decreases polyphenols in cocoa.[12, 53] While there are significant losses of polyphenols during fermentation, many of the compounds that remain have undergone other complex chemical reactions such as polymerization, condensation, etc.[12] Therefore, although the total concentration of polyphenols decreases, degradation contributes to formation of larger molecular weight compounds. Identification of these highly polymerized compounds is difficult due to analytical limitations and lack of standards.

The 4.8- and 2.6-fold decreases in total flavanols between UB/FB and UL/FL, respectively (**Figure 3.4B**), demonstrate that flavanols were more sensitive to fermentation than crude polyphenols. Previous studies have shown extensive degradation of monomeric flavanols such as catechin and epicatechin during cocoa fermentation, which the authors attribute to oxidation and polymerization of these smaller compounds.[53, 180] Albertini *et al.*[177] observed ~75% decreases in EC concentration in cocoa beans after 6 d of fermentation (the majority of losses occurring within the first 2 d) as well as larger variability of EC concentrations than total polyphenols in the fermented beans. The large losses of flavanols between unfermented and fermented samples were also demonstrated by HPLC (**Figure 3.4E**); however, these losses were not reflected as dramatically in total polyphenol losses as discussed above. It is possible that flavanols may be converted to compounds that are not measured by DMAC and other more specific methods, but which remain active in the Folin assay during these processes. We believe this to be a reason why flavanols decreased so drastically upon fermentation of cocoa samples, whereas decreases in total polyphenols were smaller.

Overall, the mDP trends between the four samples measured by thiolysis were the same for mDP (oligomers + polymers) and mDP (total flavanols). Incorporating monomeric flavanols into the mDP calculation decreased mDP values, as expected. We observed a significant increase in mDP (O+P) (2.85 to 4.16) and mDP (total flavanols) (2.04 to 3.16) from UB to FB, respectively (**Figure 3.4D)**. This is likely due to polymerization reactions as discussed above. HPLC chromatograms (**Figure 3.4E**) show relatively significant decreases in monomers and oligomers between UB and FB, which also contribute to increases in mDP. There was no significant difference in mDP (O+P) between UB/UL and UL/FL nor mDP (total flavanols) between UB, UL, and FL (**Figure 3.4D**). Assuming that the two liquors were produced from similar beans and roasted under similar conditions (which is not known), this may suggest that whatever increases in mDP that may have occurred during fermentation were then reduced through roasting, potentially due to high temperatures and presence of oxygen (comparison of liquors known to be from the same beans and roasted similarly would be needed to confirm this). Oracz *et al*.[41] observed an increase in catechin content upon roasting cocoa samples (varying with temperature and relative humidity), which may be due to the degradation of larger compounds into smaller units and epimerization of monomers. mDP data presented in this study further validate the notion that smaller compounds condense into complex, polymerized compounds during fermentation. However, during roasting, these reactions end and reactions that reduce DP begin.

Figure 3.3 shows the purple color of the UB and UL extracts, whereas FB and FL contain mostly red-brown pigments. Our results show a complete loss of monomeric anthocyanins between UL and FL and nearly a complete loss between UB and FB (**Figure 3.4C**). Previous studies have concluded that cocoa fermentation significantly reduces anthocyanins.[12, 41] Oracz *et al*.[41] studied five cocoa treatments under different fermentation conditions and determined that polyphenol oxidase reduces anthocyanins, resulting in formation of larger, brown polymers. This is evident in the color differences seen in **Figure 2**. The loss of anthocyanins highlights differences between cocoa and other fermentations, such as wine fermentation. While anthocyanin degradation is extensive in cocoa fermentation, some remain intact after wine fermentation, contributing to its red color, while others condense with grape skin tannins to form polymeric pigments.[40] This suggests that there are factors other than

microbial activities (heat, oxidation, pH, etc.) that modify polyphenol profiles during cocoa fermentations.

Due to the poor intestinal absorption of cocoa flavanols (particularly procyanidins), we believe that a major site of their activity is likely in the gut lumen, which is why we investigated their inhibition of luminal digestive enzymes. Inhibition of these enzymes has the potential to slow postprandial glucose and lipid absorption, thus blunting blood glucose spikes, slowing gastrointestinal transit, enhancing satiety, and reducing lipid accumulation, which may relate to Type-2 diabetes and obesity. While drugs such as Acarbose and Orlistat can be effective enzyme inhibitors, gastrointestinal side effects remain of major concern, which is why dietary strategies to achieve similar activities without the side effects are attractive prospects.

This work has indicated that the polyphenolic constituents of both unfermented and fermented cocoa (particularly those of cocoa liquors) are promising dietary inhibitors of α-glucosidase. UL, FL, Acarbose, FB, and UB had IC_{50} values of 90.0, 109, 158, 200, and 218 μg/mL, respectively, demonstrating that it would require lower dosages of the two liquor constituents than Acarbose to equally inhibit α-glucosidase activity. At 200 μg/mL concentrations, UL and FL were significantly better ($p<0.05$) inhibitors than Acarbose whereas UB, FB, and Acarbose had similar inhibitory capabilities. Therefore, although UB and FB had higher IC_{50} values than Acarbose, these two extracts are also capable of effectively inhibiting α-glucosidase at higher concentrations. Previous studies of α-glucosidase inhibition by natural products have concluded that inhibition is dependent on smaller, low-molecular weight phenolic compounds rather than larger polymers.[16, 136] Since we observed lower mDP values for the liquor extracts from thiolysis, this could be a reason as to why the liquors were more effective inhibitors than beans. Additionally, it is possible that certain new compounds were formed in the roasting of the liquors that were not measured by our panel of assays, but which significantly contribute to α-glucosidase inhibition. Our data suggest that cocoa constituents may serve as natural inhibitors, potentially achieving similar activities without the potential side effects of Acarbose. Additionally, we believe that the doses used *in vitro* are relatively achievable when calculated to those equivalent for *in vivo* effects. For example, the highest dose, 2000 μg/mL, would be approximately equal to 40 g cocoa product per 2 L gastric volume (1 square of baker's chocolate = 28 g).

Although Acarbose was a stronger inhibitor of α-amylase than cocoa extracts, when one-way ANOVA was performed on inhibition at 500 μg/mL, Acarbose, FB, and FL inhibited α-amylase at 71.0%, 53.6%, and 38.4% respectively, all of which were not significantly different ($p<0.05$) (FL %I data had a high standard deviation with no outliers detected; this could be why there was no difference detected compared to Acarbose by ANOVA with Tukey's *post hoc* test). This shows promise for fermented cocoa constituents as moderate inhibitors of α-amylase at high concentrations. Studies have suggested that pancreatic α-amylase inhibition depends on the presence of larger DP compounds[16, 124]. Since we observed an increase in DP as a result of fermentation, this could explain the efficacy of FB in α-amylase inhibition even though total polyphenols and flavanols decreased, as it demonstrated the highest DP of the four extracts. As for the observed efficacy of FL, it is possible that bioactive components were formed during fermentation that retained their bioactivity during roasting even though overall DP decreased.

The same observation was found in the inhibition of lipase; at 200 μg/mL, Orlistat, UB, and FB inhibited lipase 62.9%, 47.6%, 41.5%, all of which were not significantly different ($p<0.05$). This suggests that cocoa bean constituents are potentially moderate lipase inhibitors at high concentrations (**Figure 3.5 H**). It has been suggested that the presence of galloyl moieties and/or increased flavanol polymerization enhances lipase inhibition.[18, 151] We know that cocoa contains low amounts of galloylated flavanols, potentially explaining why CEs were relatively ineffective compared to Orlistat. Compared to the other samples, UB displayed the lowest IC_{25} value against lipase (**Table 3.5**). While our HPLC chromatograms suggest that UB contains large amounts of oligomers and polymers, thiolysis demonstrated a lower DP compared to FB (**Figure 3.4 D**). Therefore, we cannot correlate our UB observations with the conclusions of previous studies that larger DP results in increased inhibition of lipase. This could, however, be the reasoning behind the relative effectiveness of FB as an inhibitor since this sample showed the highest DP of all four samples. UB activity may be due simply to the large amounts of total polyphenols and flavanols (**Figures 3.4A-B**). It should be noted that we did not perform kinetic analyses on these enzymes; refer to Gu *et al.*[7] for kinetics of pancreatic lipase.

Figure 3.8 illustrates the correlation analysis between total polyphenols, total flavanols, anthocyanins, and mDP with IC_{50}/IC_{25} values of each cocoa extract. Initially we hypothesized that polyphenol decreases during fermentation and roasting would correspond with reduced inhibitory activities for all digestive enzymes tested; however, this was only demonstrated for

pancreatic lipase based on observed IC_{25} values. Overall, the only strong correlations found were between α-amlyase and total flavanols (R=0.960) and anthocyanins (R=0.962) with increasing IC_{50} as compound concentration increased (the opposite of our hypothesis, suggesting that losses of these native compounds result in improved activity against this enzyme, potentially due to formation of new bioactives). Although we saw large losses in polyphenols from fermentation and roasting, the lack of correlations in **Figure 3.8** led us to believe that these losses did not uniformly affect overall bioactivity and that other unidentified factors are involved. Upon examining the inhibition of α-amylase and lipase by unfermented, standard, and Dutch-processed cocoa liquor extracts, Gu *et al.*[7] observed significant decreases in total polyphenols between the three groups. However, inhibition remained similar between *lavado* (unfermented) and standard liquors. While differences were noticed in Dutched liquor, these observations agreed with ours in that polyphenol losses due to fermentation did not drastically affect amylase and lipase inhibition. It is possible that fermentation metabolites and/or other compounds remaining in the extracts (fiber, caffeine, etc.) could be interfering with flavanol inhibition. However, another possibility is that bioactivities were influenced by compounds formed in the condensation and oxidation reactions of processing. Dorenkott *et al.*[8] measured total polyphenols and bioactivity of cocoa fractions *in vivo* and ultimately concluded that flavanol bioactivity was not correlated with total polyphenol content, but instead with structure and mDP. **Figure 3.8** demonstrates only moderate correlations of our data between mDP and inhibition of α-glucosidase (R=0.654) and lipase (R=0.657) and no correlation with α-amylase. This led us to believe that DP may not be related to cocoa extract bioactivity as significantly as originally thought, at least for digestive enzyme inhibition.

This lack of correlations between enzyme inhibition and polyphenol content was the most novel finding of this study. It is generally accepted that bioactivity depends on higher levels of polyphenols/flavanols, thus demonstrating a proportional relationship. However, our data suggests that this is not necessarily true. In some cases, changes/losses of native polyphenols actually improve enzyme inhibition, which is opposite of what one would expect.

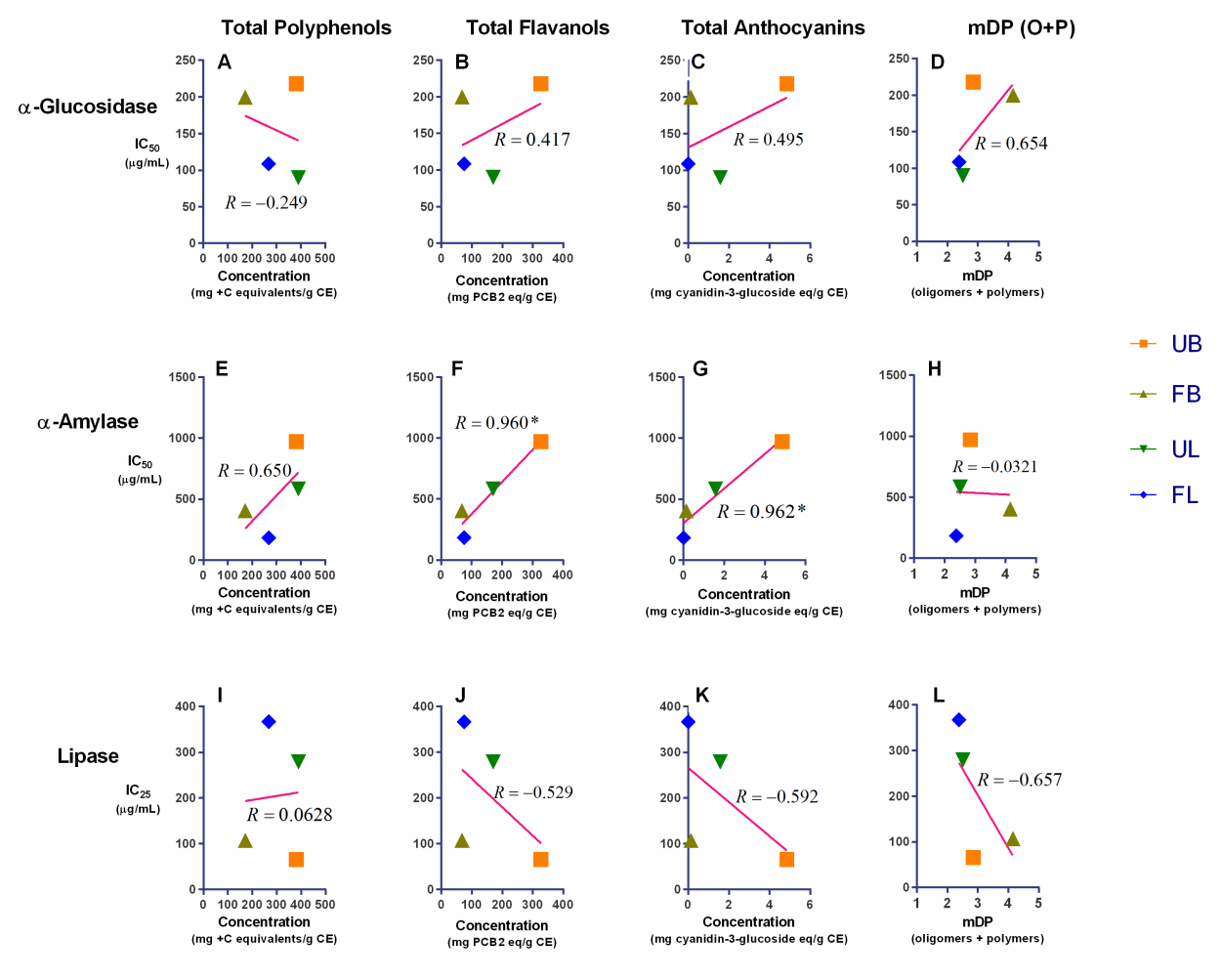


Figure 3.8 Correlation between CE chemical composition and enzyme IC_{50}/IC_{25} values. (A) Total polyphenols and α-glucosidase; (B) Total flavanols and α-glucosidase; (C) Total anthocyanins and α-glucosidase; (D) mDP and α-glucosidase; (E) Total polyphenols and α-amylase; (F) Total flavanols and α-amylase; (G) Total anthocyanins and α-amylase; (H) mDP and α –amylase; (I) Total polyphenols and lipase; (J) Total flavanols and lipase; (K) Total anthocyanins and lipase; (L) mDP and lipase. Note: for figures D, H, and L, the x-axis scale ranges from 1-5 for mDP since there cannot be an mDP value of zero. Asterisks (*) beside R values indicate slopes that are significantly non-zero ($p < 0.05$).

A potential explanation for these observations is the formation of bioactive compounds during fermentation, which is conceptually similar to tea fermentation (flavanols → theaflavins → thearubigins → theabrownins). Upon extreme condensation, these compounds can form large, complex structures that ultimately resemble lignin and fiber, potentially acting in similar ways, binding proteins and improving enzyme inhibition. Previous studies have shown that these compounds possess beneficial bioactivities. Honda and Hara[172] suggested anti-diabetic effects of tea theaflavins via an intestinal glucosidase mechanism and Gong *et al.*[173] demonstrated lipid-lowering effects of tea theabrownins in rats. Although tea and cocoa undergo very different fermentation processes, (predominantly microbiological in cocoa, predominantly polyphenol oxidase in tea) we believe that the biotransformation of polyphenolic compounds may be conceptually similar, generating large complex bioactive compounds that contribute to losses of total polyphenols and flavanols but which may preserve (or potentially enhance) bioactivity. Similarly, complex products formed during roasting may preserve or enhance activity. The presence of complex condensation compounds in cocoa has not yet been explored, as they pose analytical challenges for speciation and quantification. However, future studies could measure these compounds as broad classes based on their affinities for polar versus non-polar extraction and spectrophotometric properties as utilized by Zou *et al.*[181] We believe formation of these (or similar) compounds to be a probable mechanism occurring in cocoa fermentation and roasting, posing the opportunity to shift focus from bioactivity based solely on flavanols to inclusion of more complex compounds.

In conclusion, the research from this chapter has demonstrated that the constituents of unfermented and fermented cocoas are promising inhibitors of α-glucosidase as well as moderate inhibitors of α-amylase and lipase in relation to blood glucose absorption and fat accumulation. Although large losses in polyphenols, flavanols, and anthocyanins were observed from fermentation, we believe that there are other factors (potentially fermentation and roasting products) affecting cocoa bioactivity. The most novel finding of this research was that enzyme inhibiting bioactivities of cocoa were not necessarily proportional to polyphenol concentrations, which goes against the widely accepted dogma that bioactivity is improved by higher concentrations of polyphenols. Based on these results, further research would be beneficial in the exploration of cocoa components resulting from different processing conditions in the prevention of Type-2 diabetes and obesity.

Chapter 4

Assessment of Dipeptidyl Peptidase-IV Inhibition, Protein-Binding Abilities, and Presence of Other Potentially Bioactive Compounds in Cocoa Products Processed to Varying Degrees

Introduction

The potential use of cocoa (*Theobroma cacao*) products has been explored recently as a strategy to aid in prevention and/or amelioration of diseases such as diabetes and obesity due to the presence of bioactive flavanol (flavan-3-ol) compounds.[7, 8] Cocoa contains the highest concentration of flavanols (on a per-weight basis) compared to any other food; these compounds include (+)-catechin (+C), (–)-catechin (–C), (–)-epicatechin (EC), and their oligomers and polymers known as the procyanidins (PC) (**Figure 4.1**). The high concentration of flavanols has been the driving factor behind the interest in a multitude of prospective beneficial bioactivities[9, 10, 56] of cocoa.

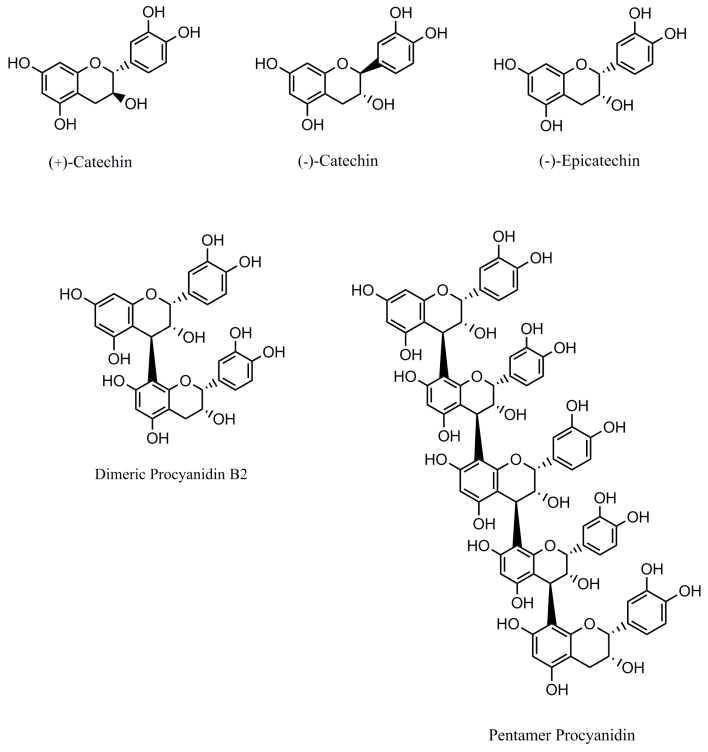


Figure 4.1. R*epresentative structures of flavanols commonly found in cocoa*

Glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) are gastrointestinal hormones (known as incretins) that stimulate insulin release in response to glucose ingestion, increase satiety, slow gastric emptying, and promote glucose homeostasis, which are beneficial activities for the prevention and amelioration of Type-2 diabetes.[116, 122] GLP-1 and GIP are rapidly degraded by dipeptidyl peptidase-IV (DPP4), resulting in extremely short circulating half-lives (1-1.5 min for GLP-1 and about 7 min for GIP).[118-120] Once inactivated, insulin secretion is reduced and glucose homeostasis is negatively affected. Various pharmaceutical DPP4 inhibitors are commercially available and have shown effectiveness in maintaining elevated postprandial GLP-1 and GIP activity after 12 weeks of treatment in patients with impaired glucose tolerance.[112, 155] Products rich in flavanols, such a procyanidin-rich grape seed extracts have been shown to decrease DPP4 activity and improve glucose control in human intestinal cells and in diet-induced obese rats,[153] which lead us to believe that cocoa procyanidins could act in a similar manner. Cocoa liquor procyanidins have demonstrated anti-hyperglycemic effects *in vitro* and *in vivo*.[182, 183] Of particular interest is the finding by Yamashita *et al*[121] that tetrameric PC Cinnamtannin A2 (CinA2; isolated and purified from cacao liquor) increased plasma insulin and GLP-1 secretion *in vivo* 60 min after oral administration in mice. This effect may be due to inhibition of DPP4 by CinA2, thus preventing the rapid breakdown of GLP-1 and preserving its insulin-increasing activities. Therefore, one objective of this study was to determine the DPP4 inhibitory activities of cocoa extracts (CEs) with different degrees of processing.

In the previous chapter, we demonstrated the ability of these CEs to inhibit luminal digestive enzymes. We determined the correlations between inhibitory activities and properties such as Folin values, total flavanols, anthocyanins, and flavanol mean degree of polymerization (mDP). Our findings suggest that total polyphenol and/or flavanol levels do not generally correlate well with inhibition of digestive enzymes. Pursuing this line of research, we strove to further examine and quantify other functional and compositional properties of these products as a means to explain differences in observed biological activities.

Astringency is a desirable characteristic of foods or beverages rich in flavanols, such as red wine, dark chocolate, tea and cider. The physical sensation of astringency is caused by the binding of salivary proteins by polyphenols in a food or beverage.[184] Since digestive enzymes are proteins, we hypothesized that non-specific protein-binding abilities of cocoa products could

partly explain their enzyme inhibiting abilities, in addition to potential mixed and competitive digestive enzyme inhibition mechanisms involved, as reported by Gu *et al*.[7]

Fermentation and roasting of cocoa beans, common processes in cocoa manufacturing, are known to decrease their levels of naturally present polyphenols.[12, 53, 74] While it remains unclear how these changes in polyphenol composition affect their activities *in vivo*, it has been generally assumed that higher levels of polyphenols are essential for optimal bioactivity.[177] The CEs utilized in our previous and current studies represent different levels of cocoa processing including unfermented, fermented, unroasted, and roasted cocoa products. We previously demonstrated that cocoa extract bioactivity did not necessarily depend on total polyphenol or flavanol content, and that the effects of fermentation and roasting varied depending on the enzyme evaluated.

Building upon our previous study, we aimed to quantify other potentially bioactive compounds besides flavanols that may be formed during cocoa processing. Cocoa processing improves palatability in part by decreasing bitterness. As flavanol mDP increases, bitterness decreases.[185] Highly polymerized, catechin-containing compounds (oxidative polymers) are commonly found in products such as fermented tea (oolong, black and pu-erh tea made from *Camellia sinensis* leaves).[186] Theaflavins are dimers formed by enzymatic oxidation of flavanol monomers during tea fermentation.[167, 168] It is thought that even more complex species (thearubigins/theabrownins) are formed by the polymerization, condensation, and oxidation of theaflavins with catechins and may incorporate things such as proteins, lipids, and caffeine.[169, 187] Previous studies have reported anti-diabetic and lipid-lowering effects of these compounds.[172, 173] Although cocoa and tea undergo different fermentation processes, (and theaflavins are produced from enzymatic fermentation of flavanols with epigallocatechin and epigallocatechin gallate,[188] which are not present in appreciable quantities in cocoa[12, 189]), conceptually it is possible that similar highly polymerized compounds may form during processing of cocoa, possibly retaining or improving bioactivity of the raw cocoa.

The objectives of this study were to explore the DPP4 inhibitory activities of cocoas with distinct degrees of processing, as well as examine their levels of individual flavanols, levels of oxidative polymers, and protein binding activities. We then strove to potentially correlate our previous digestive enzyme inhibition with protein binding ability and/or presence of these compounds.

Materials and Methods

Chemicals and Standards. Trizma-HCl, Ile-Pro-Ile, and citric acid were obtained from Sigma Aldrich (St. Louis, MO). DPP4 (human, recombinant) was obtained from Enzo Life Sciences (Farmingdale, NY). GP-pNA chromogenic substrate was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Sigma-Aldrich (St. Louis, MO). Albumin bovine was obtained from Spectrum Chemical Manufacturing Corporation (New Brunswick, NJ). All solvents used were ACS grade or higher.

Cocoa Samples. Four cocoa samples were obtained and extracted as described in the previous chapter. The same CE samples utilized in our first study were used in these assays. As described previously, unfermented cocoa beans (UB) and fermented cocoa beans (FB) were harvested in Malaysia and donated by Cargill, Inc. (Minnetonka, MN). FB had undergone ~4 d of fermentation. Unfermented cocoa liquor (UL) and fermented liquor (FL) were donated by The Hershey Co. (Hershey, PA). Liquor is the solid mass that results from roasting and grinding of cocoa beans, and which contains both cocoa solids and cocoa butter. Polyphenolic profiles, including total polyphenols, total flavanols, monomeric anthocyanins, and flavanol mean degree of polymerization (mDP) of the four extracts were quantified previously. These values are shown in **Table 4.1**.

Table 4.1. *Chemical compositions of cocoa extracts and original cocoa products (expressed as mean value)*

Sample[a]		Total Polyphenols		Total flavan-3-ols (mg PCB2 eq/g)	Total anthocyanins (mg cyanidin-3-glucoside eq./g)	mDP[b]	
		(mg +C eq/g)	(mg GAE/g)			(Oligomers + polymers)	(Total flavan-3-ols)
Extract	**UB**	381	424	327	4.85	2.85	2.04
	FB	171	232	67.7	0.130	4.16	3.16
	UL	390	432	170	1.58	2.51	1.92
	FL	268	321	64.7	0	2.39	2.00

[a]UB; unfermented beans, FB: fermented beans, UL: unfermented liquor, FL: fermented liquor
[b]mDP: mean degree of polymerization/thiolysis value (oligomers + polymers does not incorporate monomers present prior to thiolysis into the mDP calculation)

Inhibition of Dipeptidyl Peptidase-IV (DPP4). Inhibition of DPP4 was measured according to the method utilized by Lin *et al.*[15] with modifications. DPP4 (human, recombinant) was diluted with 100 mM Tris buffer (pH 8) to a final concentration of 2 mU/mL. Cocoa extracts were dissolved in DMSO and further diluted with distilled deionized water so that each sample tube contained 10% DMSO (0-8000 μg/mL CE solutions). In a 96-well plate, 50 μL CE solution (n=6; 0-2000 μg/mL final concentration in the reaction with 2.5% DMSO final concentration) and 50 μL of the DPP4 solution were combined. A second plate was prepared with the same cocoa extract solutions, replacing the enzyme solution with 50 μL of 100 mM Tris buffer (pH 8) in order to account for the absorbance of the pigments naturally present in the substrate and extracts. The plates were covered and incubated at 37°C for 10 min. Then, 50 μL 1 mM Gp-pNA chromogenic substrate was added to each well, followed by 50 μL 100 mM Tris buffer (pH 8). The plates were incubated at 37°C for 60 min. After incubation, 50 μL 3% acetic acid was added to each well to end the reaction and absorbance was read at 405 nm. The 0 μg/mL sample was used as the negative control to measure full (uninhibited) DPP4 activity. Ile-Pro-Ile (Diprotin A) was diluted to the same concentrations as the cocoa extracts using DMSO and distilled water and used as the positive control. The different concentrations of cocoa extract solutions were compared to the controls and expressed as % DPP4 activity. Activity was calculated as follows:

$$\%\ DPP4\ Activity = \frac{(A_{I,S,E} - \bar{A}_{I,S})}{(\bar{A}_{S,E} - \bar{A}_{S})}\ x\ 100$$

Where:

$A_{I,S,E}$ = the individual absorbance value of the product of the inhibitor, substrate, and enzyme at each inhibitor dose

$\bar{A}_{I,S}$ = the average absorbance value of the inhibitor and substrate (no enzyme) reaction

$\bar{A}_{S,E}$ = the average absorbance value of the substrate and enzyme reaction without inhibitor (0 μg/mL)

$\bar{A}_{S}$ = the average absorbance value of the substrate without enzyme and inhibitor

Individual Flavanol Analysis. Flavanols were quantified by HPLC with electrochemical detection (ECD) as described previously.[14] The HPLC system used consisted of two LC-20AD

pumps, a SIL-20AC autosampler (Shimadzu Scientiffic, Kyoto, Japan), and an ESA 5500 coulochem electrode array system (CEAS; Chelmsford, MA). ECD potentials were –100, 100, 300, and 500 mV for the CEAS and chromatographic separation was done on a 150 nm x 4.6 mm, 5 μm, Supelcosil LC-18 column (Supleco, Bellefonte, PA). Mobile phase solvent A was 30 mM sodium phosphate monobasic buffer, 1.75% acetonitrile, and 0.125% tetrahydrofuran (pH 3.35) and solvent B was 15 mM sodium phosphate monophosphate buffer, 58.5% acetonitrile, and 12.5% tetrahydrofuran (pH 3.45). Mobile phase B began at 4% with isocrastic flow for 7 min, then increased to 17% at 25 min, 33% at 45 min, then 98% at 60 min, where it was held for 3 min before re-equilibration to 4%. Flow rate was 1.0 mL/min and column temperature was 35°C for the entire analysis. Cocoa extracts (n=3) were dissolved in DMSO and diluted 10-fold with 0.2% ascorbic acid in deionized water. Samples were filtered through 0.45 μM PTFE filters (VWR Internations, Radnor, PA) and 30 μL was injected into the HPLC system. ECD quantification was performed at100 mV detector potential for all compounds. Flavanols were quantified by DP with the exception of monomeric catechins (catechin and epicatechin were quantified individually).

Polymeric Pigments. Measurement of polymeric pigments as an indication of oxidative polymers in cocoa was performed based off of the methods used by Wang *et al*.[171] and Zou *et al*.[181] These compounds were crudely separated into different classifications based on their affinities for polar versus non-polar extraction solvents and spectrophotometric properties. Ethyl acetate, butanol, 95% ethanol, and distilled deionized water were utilized as extraction solvents. In the method utilized by Zou *et al*,[181] theaflavins corresponded to those compounds soluble in ethyl acetate, thearubigins were soluble in butanol, and theabrownins in 95% ethanol, demonstrating an increase of compound size with increase solvent polarity. Wang *et al* states that theabrownins are soluble in water and not non-polar solvents,[171] which is why we added a water extraction to separate out even larger, water-soluble compounds. While the specific polymeric pigment compounds in cocoa are likely different than those found in tea, the principle of compound separation based on size and polarity is similar. To fractionate based on polarity, 50 mg cocoa extract (n=4) was measured out and combined with 200 μL ethyl acetate. The solutions were vortexed and sonicated to mix, then centrifuged at 17,000 x g for 5 min and the supernatant retained. Extraction with ethyl acetate was repeated two more times, pooling the

supernatants of each individual CE sample. The entire extraction procedure was repeated using butanol, 95% ethanol, then distilled deionized water sequentially, utilizing the same CE pellet throughout all extractions once previous supernatants had been removed. Water extracts were then diluted 10x and EtOH extracts 5x in order to be within the measureable absorbance range. 250 μL of each pooled supernatant (n=4 for each CE sample and solvent) was transferred to a 96-well plate and absorbance was read at 380 nm. Flavanols generally absorb ~280 nm due to the aromatic rings[190], but not at wavelengths >300 nm[191], where flavanol oxidation/condensation products that may be present in fermented/roasted products could absorb. Theaflavins have maximal absorbance at 380 nm[192, 193], which is why we used this area of the spectrum to measure oxidative polymers. We believe that at this wavelength, flavanols will not absorb, but functional groups associated with oxidation products similar to theaflavins and more complex compounds can be measured and interpreted as oxidative polymers.

The entire extraction procedure was then repeated using the original cocoa samples rather than cocoa extracts. 250 mg cocoa sample (n=4) was first frozen in liquid nitrogen and ground into a powder. The extraction procedure remained the same as that used for the cocoa extracts except that solvent volume utilized was 1 mL instead of 200 μL. Water extracts were diluted 2x and EtOH extracts 2x in order to be within the measureable absorbance range. 250 μL of each pooled supernatant was transferred to a 96-well plate and absorbance was read at 380 nm.

Protein Precipitation. Protein precipitation activities of the cocoa extracts were assessed based on the procedure utilized by Watrelot *et al.*[161] A citrate/phosphate buffer (pH 3.8) was first prepared by combining 0.1 M citric acid and 0.2 M sodium phosphate solutions. A 2 mg/mL BSA solution in citrate/phosphate buffer (pH 3.8) was prepared as the working protein solution. Cocoa extract samples (n=4) were dissolved in DMSO and further diluted with the citrate/phosphate buffer (pH 3.8) so that each sample tube contained 10% DMSO (0-4000 μg/mL CE solutions). In the top four rows of a 96-well plate, 100 μL 2 mg/mL BSA in buffer solution was combined with 100 μL diluted cocoa extract solution (n=4; 0-2000 μg/mL in reaction with 5% DMSO final concentration). (Note: prior to performing the assay, a test was performed to confirm that a 5% DMSO final concentration would not interfere with protein aggregation). In the bottom four rows of the plate, 100 μL citrate/phosphate buffer (pH 3.8) was combined with 100 μL diluted cocoa extract solution (0-2000 μg/mL in reaction, n=4) as a negative control. The

contents of the plates were mixed and absorbance was read at 650 nm to quantify turbidity. Average negative control values for each concentration were subtracted from the protein/CE values in order to account for absorbance values (from the pigments) due to cocoa extract alone. Haze measured at this absorbance is proportional to protein bound and precipitated out of solution by the cocoa extracts.

Data Analysis and Statistics. If necessary, Dixon's Q-test (α=0.05) was performed in order to detect and eliminate outliers from data sets. A limitation of this study was that the beans and liquors were not obtained and produced from the same source. Additionally, we did not know whether the liquors were made from the same beans or if the liquors were roasted under the same parameters. For discussion purposes, the four extracts were considered four independent treatment groups and assumed that the liquors were produced from the same source and under the same roasting conditions. Levels of individual flavanols, protein binding at individual concentrations, and pigment absorbance values grouped by extraction solvent were analyzed by one-way ANOVA; if a significant treatment effect was found, Tukey's HSD *post hoc* test was performed to determine differences between treatment means. Concentration at which DPP4 activity was inhibited 25% (IC_{25}) by Ile-Pro-Ile was calculated using four parameters sigmoidal analysis. Linear regression was employed to estimate IC_{25} values for CE samples, since the data did not fit a sigmoidal curve. IC_{25} was utilized as opposed to IC_{50}, which is more commonly reported, due to the fact that cocoa samples did not achieve 50% inhibition. Enzyme inhibition values (%I) at individual concentrations were analyzed by one-way ANOVA; if a significant treatment effect was found, Tukey's HSD was then performed to determine differences between treatment means. Statistical significance was defined as $p < 0.05$. Analyses were performed on Prism v6.0f (GraphPad, La Jolla, CA).

Results

Inhibition of DPP4. Cocoa extracts slightly inhibited DPP4 in dose-dependent manners (**Figure 4.2**) compared to the + control (Ile-Pro-Ile), which was a very effective inhibitor. IC_{25} values for Ile-Pro-Ile, FB, UB, FL, and UL were 4.82, 1585, 2135, 2217, and 2871 μg/mL, respectively (**Table 4.2**). Based on statistical analyses, the order of %I at both 1000 and 2000 μg/mL was Ile-Pro-Ile > FB = FL = UB = UL. Maximum %I by UB, FB, UL, and FL were 22.4%, 30.6%, 15.7%, and 22.8% at 2000 μg/mL concentrations, respectively.

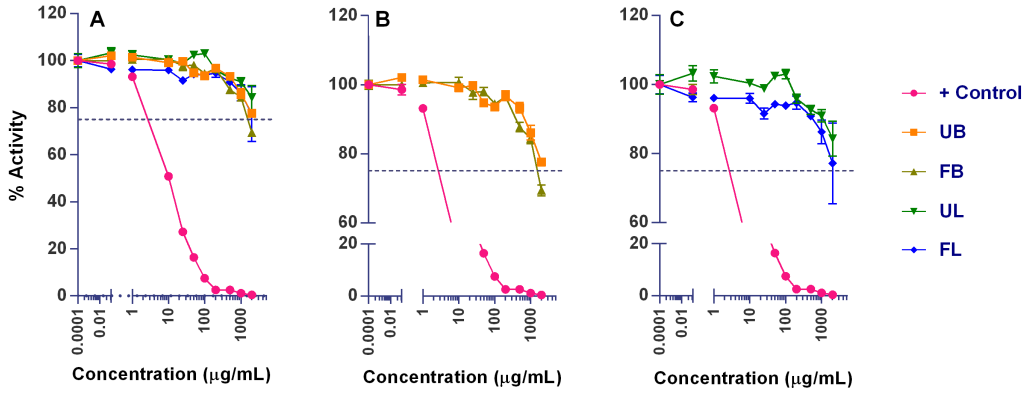


Figure 4.2. DPP4 % Activity for (A) all cocoa extracts versus the Ile-Pro-Ile control; (B) unfermented and fermented bean CEs; and (C) unfermented and fermented liquor CEs. Dotted line represents 75% DPP4 activity (i.e 25% inhibition). Activity at 0 μg/mL was plotted as 0.0001 μg/mL in order to facilitate graphing on a logarithmic scale. Note: % Activity scales for figures B and C differ from that of figure A.

Table 4.2. Enzyme inhibition parameters for cocoa extract samples and positive controls

Enzyme	Parameter[a]		Inhibitor[c]				
			+ Control[b]	UB	FB	UL	FL
DPP4	IC_{25} (μg/mL)		4.82	2135	1585	2871	2217
	R^2		0.993	0.902	0.966	0.859	0.904
	% I	10 μg/mL	49.2 ± 0.690[a]	0.84 ± 0.585[bc]	–0.7 ± 1.54[c]	–0.4 ± 0.948[bc]	4.03 ± 1.48[b]
		1000 μg/mL	98.8 ± 0.520[a]	13.9 ± 2.13[b]	15.6 ± 1.58[b]	8.98 ± 1.83[b]	13.7 ± 3.47[b]
		2000 μg/mL	99.6 ± 0.218[a]	22.4 ± 0.973[b]	30.6 ± 1.59[b]	15.7 ± 5.06[b]	22.8 ± 11.7[b]

[a]IC_{25}: concentration resulting in 25% inhibition compared to uninhibited activity, R^2: goodness of fit for the chosen model, %I: % inhibition of enzyme activity at the indicated concentration (those near calculated IC_{25} values)
[b]Positive inhibitor control: Ile-Pro-Ile (Diprotin A)
[c]For % inhibition, values with different letter superscripts in the same row are significantly different (one-way ANOVA, Tukey's HSD post hoc test between all means, $p < 0.05$)

Individual Flavanol Analysis. Detected levels of each compound (C, EC) or group of compounds (DP 2-7) are shown in **Table 4.3** and **Figure 4.3**. Between the four cocoa extracts, levels of each compound varied. Overall, epicatechin and DP 6 were present in the highest amounts among the extracts. **Figure 1** demonstrates that UB and/or UL contained significantly higher levels of each compound than the fermented samples (except for DP 7 where FL was not significantly different than UB).

Table 4.3. Quantification of individual flavanols based on degree of polymerization (expressed as mean ± SEM)

Compound (mg/g extract)	Sample[a]			
	UB	FB	UL	FL
Catechin	0.349 ± 0.0148^{b}	0.0901 ± 0.0348^{a}	0.796 ± 0.0557^{c}	0.409 ± 0.00668^{b}
Epicatechin	60.6 ± 10.1^{c}	22.4 ± 0.443^{ab}	48.3 ± 5.90^{bc}	12.7 ± 0.183^{a}
DP 2	16.8 ± 1.26^{b}	8.70 ± 0.216^{a}	21.9 ± 2.07^{b}	9.60 ± 0.144^{a}
DP 3	0.314 ± 0.0545^{a}	0.191 ± 0.0842^{a}	4.39 ± 0.170^{c}	2.40 ± 0.0625^{b}
DP 4	18.3 ± 2.05^{c}	3.91 ± 0.165^{ab}	8.83 ± 0.649^{b}	2.13 ± 0.171^{a}
DP 5	10.5 ± 0.319^{c}	3.05 ± 0.202^{a}	7.13 ± 0.445^{b}	1.59 ± 0.00673^{a}
DP 6	78.4 ± 13.8^{c}	24.4 ± 1.14^{ab}	47.9 ± 3.63^{bc}	2.19 ± 0.584^{a}
DP 7	0.350 ± 0.0461^{c}	0.0784 ± 0.0334^{a}	0.151 ± 0.00895^{ab}	0.312 ± 0.0242^{bc}

[a]Values with different letter superscripts in the same row are significantly different (one-way ANOVA, Tukey's HSD post hoc test between all means, $p < 0.05$)

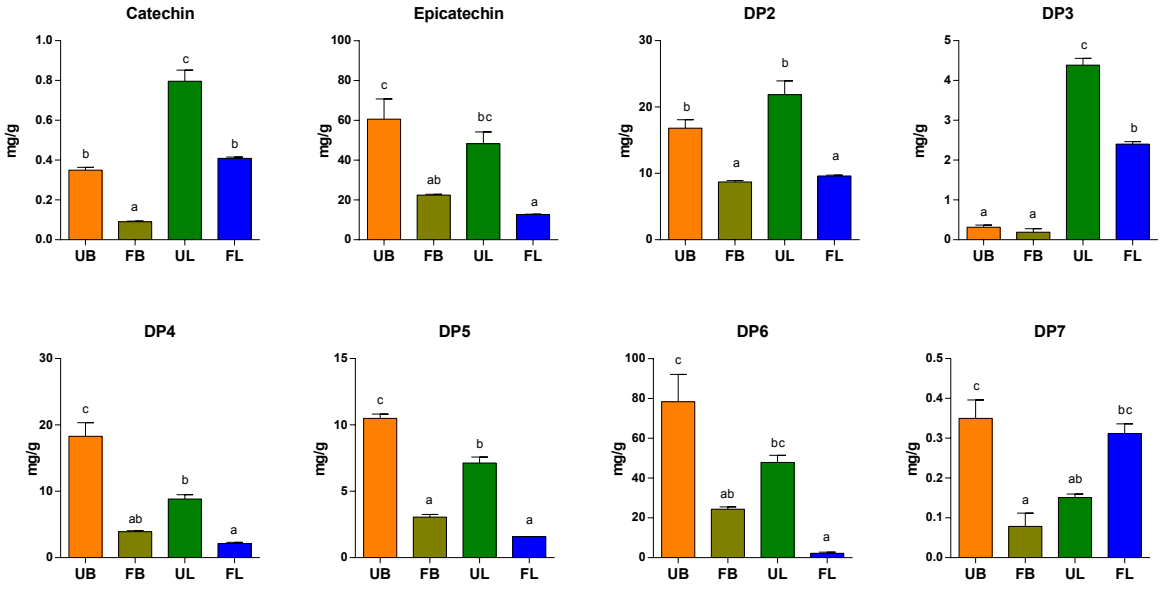


Figure 4.3. Levels of flavanol compounds in each extract. Values with different letter superscripts are significantly different (one-way ANOVA, Tukey's HSD post hoc test between all means, $p < 0.05$).

Polymeric Pigments. Levels of compounds measured as oxidative polymers (compounds soluble in ethyl acetate, butanol, 95% EtOH, and water, with absorbance at 380 nm) in the cocoa extracts are shown in **Figure 4.4**. In the method utilized by Zou *et al*[181], theaflavins corresponded to those compounds soluble in ethyl acetate, thearubigins were soluble in butanol, and theabrownins in 95% ethanol, demonstrating an increase of compound size with increased solvent polarity. We added a water extraction to separate out even larger, water-soluble compounds. However, it is also possible that smaller microbial metabolites and phenolic acids could be present in the water extract. While the specific oxidation/condensation compounds in cocoa are likely different than those found in tea, the principle of compound separation based on polarity is similar. Overall, the differences in levels of each compound type due to extent of processing showed different trends. There were significant increases in ethyl acetate- and water-soluble compounds in the CEs as level of processing (fermentation and roasting) increased (FL > UL = FB = UB). UB and UL had higher amounts of ethanol-soluble compounds than FB and FL. Butanol-soluble compounds were highest in UL compared to the other three extracts.

Since our bioactivity studies were performed utilizing cocoa extracts, we were more concerned with their levels of oxidative polymers. However, we also wanted to measure these compounds in the original, non-extracted cocoa samples as well to see how they were different (**Figure 4.5**). When comparing extracts to original products, levels of each compound type varied. The original cocoa products contained higher amounts of the largest, water-soluble compounds than the extracts. Water-soluble compounds were highest in the FL extract, yet UB contained the highest amount (about 0.5-fold higher than FL CE) compared to any of the other samples.

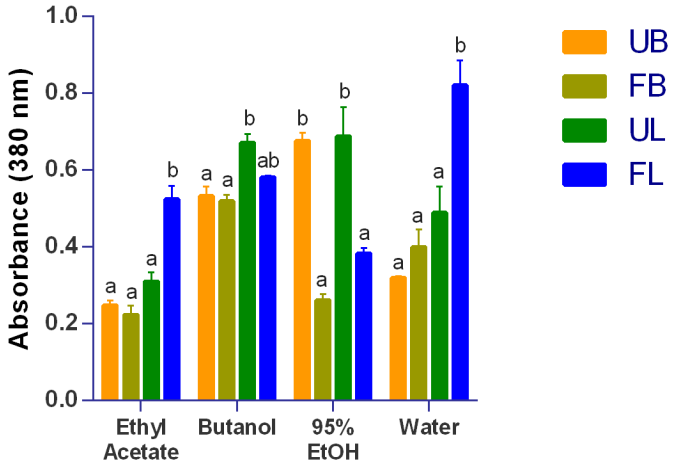


Figure 4.4. Absorbance values read at 380 nm for compounds soluble in Ethyl Acetate, Butanol, 95% Ethanol, and water present in the four CEs extracted with 70:28:2 acetone:water:acetic acid. Relative levels of each type of compound are roughly correlated to different oxidative polymers present. Values with different letter superscripts within the same solvent grouping are significantly different (one-way ANOVA, Tukey's HSD post hoc test between all means, $p < 0.05$) Note: 95% ethanol extracts were diluted 10x and water extracts were diluted 5x to be within the absorbance range.

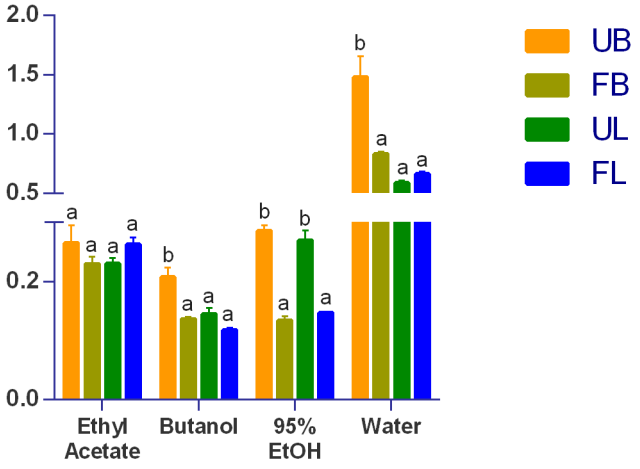


Figure 4.5. Absorbance values read at 380 nm for compounds soluble in Ethyl Acetate, Butanol, 95% Ethanol, and water present in the four original, non-extracted cocoa beans and liquors (Note differences in y-axis scales). Relative levels of each type of compound are roughly correlated to different oxidative polymers present. Values with different letter superscripts within the same solvent grouping are significantly different (one-way ANOVA, Tukey's HSD post hoc test between all means, $p < 0.05$) Note: 95% ethanol and water extracts were diluted 2x with the same solvent.

Protein Precipitation. Protein binding abilities (as measured by haze formation due to precipitation) of the four cocoa extracts are represented in **Figure 4.6**. Overall, CEs bound and precipitated the BSA at the higher concentrations (>100 μg/mL). At 1000 and 2000 μg/mL concentrations, binding ability of the extracts was UL > UB = FL > FB according to our statistical analysis.

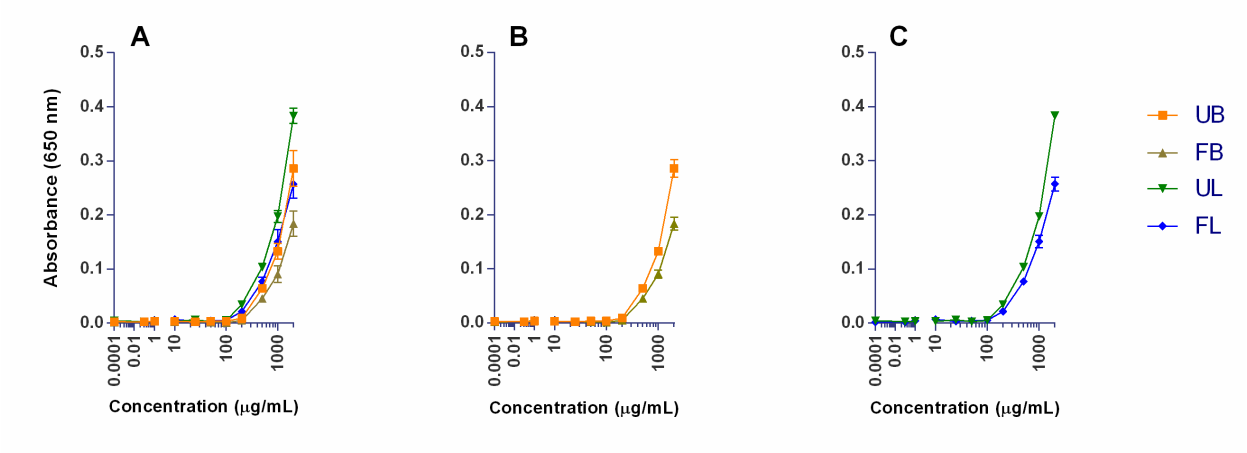


Figure 4.6. Protein binding measured as haze absorbance at 650 nm precipitated by (A) all cocoa extracts; (B) unfermented and fermented bean CEs; and (C) unfermented and fermented liquor CEs. Precipitation at 0 μg/mL (– control) was plotted as 0.0001 μg/mL in order to facilitate graphing on a logarithmic scale.

Discussion

We previously demonstrated that cocoa extracts possess potential activities as digestive enzyme inhibitors; however, these bioactivities were not strongly correlated to their differences in polyphenol levels as determined by the Folin-Ciocalteu, flavanol [by dimethylaminocinnamaldehyde (DMAC) assay], and anthocyanin pH differential methods. The current study evaluated other properties of these extracts (individual flavanol concentrations, protein binding capabilities and presence of other bioactive components) that may better explain their enzyme inhibition activities. Additionally, to the best of our knowledge, this is the first study examining the ability of cocoa extracts to inhibit DPP4, even though it showed to only slightly inhibit the enzyme. The majority of DPP4 inhibition studies utilize pharmaceuticals or grape seed extract, therefore, research on the use of other potential inhibitors present in the diet was warranted. Furthermore, we believe this to be the first study to examine potential correlations between bioactivity and oxidative polymer compounds found in cocoa, potentially shifting some of the focus from flavanols as cocoa's main bioactive components.

The IC_{25} values in **Figure 4.7** represent how fermentation and roasting affected the abilities of the CEs to inhibit DPP4. The IC_{25} values increased as follows: FB < UB < FL < UL, indicating that fermentation improved and roasting decreased inhibition since non-roasted samples (beans) had the lowest IC_{25} values and between the two sample sets, fermented samples were lower than unfermented. **Figure 4.2** demonstrates the effectiveness of the CEs and Ile-Pro-Ile control in the inhibition of DPP4. When one-way ANOVA with Tukey's HSD test was performed on %I at individual concentrations, the + control was significantly better than the CEs at all concentrations. At higher concentrations (1000 and 2000 ppm), the four CEs were not significantly different, indicating that equal amounts of any of the CEs would inhibit the enzyme similarly at high doses.

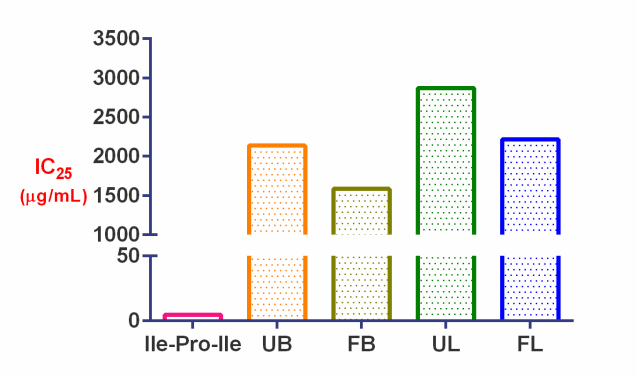


Figure 4.7. Comparison of IC_{25} values to visualize effects of fermentation and roasting on inhibition of DPP4.

Although the CEs were not as effective inhibitors of DPP4 compared to the positive control, these data do suggest some promise for the use of cocoa products as complimentary or supplementary products to help increase circulating incretin levels and activities. A recent study by Fan *et al*.[157] isolated anthocyanins from berry wines and other phenolic compounds commonly found in citrus, berry, grape, and soybean and assessed their DPP4 inhibitory activities utilizing an inhibition kit. The researchers found that anthocyanins from a blueberry-blackberry wine and compounds such as resveratrol, luteolin, apigenin, and flavone strongly inhibited DPP4 (all of which had lower IC_{50} values than the Ile-Pro-Ile positive control). Of particular interest, however, is their finding that isolated catechin and epicatechin, compounds commonly found in high concentrations in cocoa, were among compounds that had no DPP4 inhibitory activity.[157] However, previous studies have shown ~70% inhibition of commercial DPP4 with 200 ppm grape seed procyanidin extract[153] and ~40% inhibition at 500 ppm doses of different extracts from small-leaf grapes (*Vitis thunbergii* var. *taiwaniana*).[15] These results demonstrate moderate inhibition of DPP4 by grape-derived products known to contain monomeric flavanols such as catechin and epicatechin. It is possible that these inhibitory activities could be dependent on the presence of galloyl groups found in grape polyphenols, of which cocoa is known to contain low amounts.[12, 189] Therefore, DPP4 activity seems to be largely dependent on the polyphenolic structures of the natural inhibitors.

The varying trends of individual compound levels in the extracts (**Figure 4.3**) demonstrate that each of the flavanols was affected differently by fermentation and roasting of the cocoa samples. While the unfermented samples generally contained higher concentrations of

compounds that the fermented, there are some cases in which a fermented sample was either higher than (DPs 3 and 7 in FL) or not significantly different from (C, EC, DPs 4 and 6) an unfermented sample. Overall, it appears as though fermentation decreases concentrations of all individual compounds (except DP 7 between liquors). These data also indicate to us that roasting might increase levels of trimers and potentially DP7 compounds. The finding that different DP respond distinctly to processing conditions such as roasting, fermentation, and alkalization has been noted previously.[12, 14, 194]

Figure 4.8 demonstrates correlation between enzyme inhibition and concentrations of individual flavanols or flavanol DP. Upon performing linear regression, a strong, negative correlation would indicate improved inhibition due to increase concentration of a compound. However, linear trends differed greatly as a result of these analyses, with only a few negative correlations. These were found between α-glucosidase IC_{50} with catechin (R= –0.769) and DP 3 compounds (R= –0.945) suggesting that higher concentration of these relatively smaller compound classes improve α-glucosidase inhibition. This agrees with previous research suggesting that presence of smaller compounds are beneficial for inhibition of this enzyme.[16, 136] Similarly, moderate negative correlation was found between lipase IC_{25} with epicatechin (R= –0.522), DP 4 (R= –0.622), DP 5 (R= –0.562), and DP 6 (R= –0.672) compounds. This suggests that relatively larger compounds (as well as EC) moderately help to improve lipase inhibition, which has been reported previously.[151]

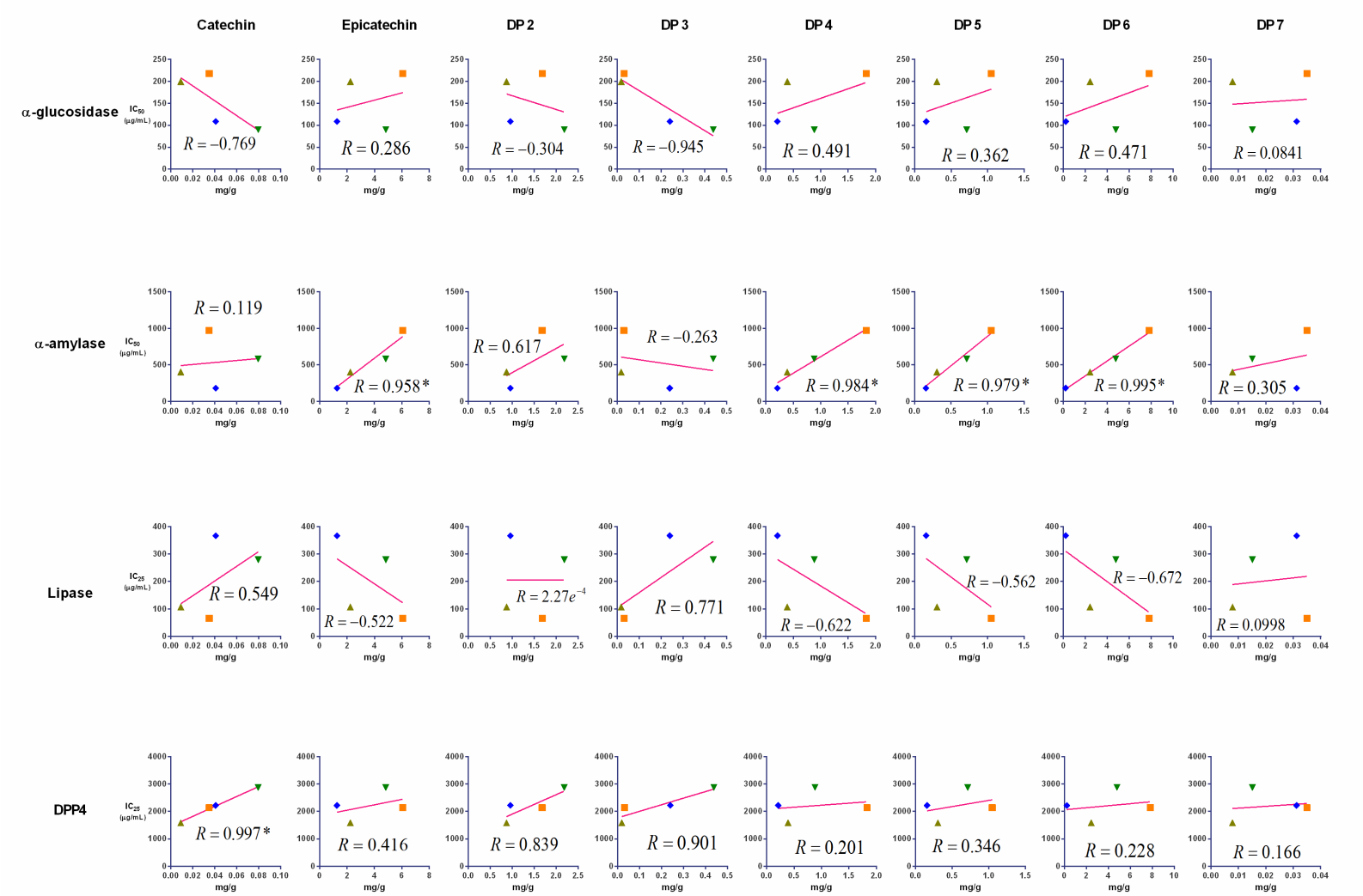


Figure 4.8. Correlations between individual flavanol concentration and enzyme IC values. Graphs demonstrate linear regression analysis with strong, negative lines indicating improved inhibition due to increased compound concentration. Asterisks (*) beside R values indicate slopes that are significantly non-zero ($p < 0.05$).

Conversely, we saw several strong (significantly non-zero slopes; $p<0.05$), positive correlations between some of the enzymes and individual compounds. α-amylase IC_{50} values had a strong association with epicatechin (R=0.958), DP 4 (R=0.984), DP 5 (R=0.979), and DP 6 (R=0.995) whereas DPP4 was strongly correlated to catechin concentration (R=0.997). These analyses suggest that as the concentration of these individual compounds increase, inhibition of the respective enzyme is hindered, which is the opposite of what we had originally hypothesized. It is unclear as to why these relationships were observed, however, we believe this to be a novel finding as it is generally thought that bioactivity would be improved as flavanol concentrations increase. Furthermore, outside of catechin and DP 3 for α-glucosidase, there do not appear to be specific compounds, or DPs, whose concentrations appear to positively drive enzyme inhibition. However, increasing concentrations of epicatechin and oligomers with DP 4-6 appear to inhibit α-amylase. The mechanisms behind these observations warrant further consideration. These results further validate our previous data suggesting that enzyme inhibition are generally not proportional to concentrations of flavanols, even when compounds are quantified individually or by DP.

Presence of compounds tentatively identified as oxidative polymers were representatively measured based on compound solubility in ethyl acetate, butanol, 95% ethanol, and water and absorbance at 380 nm. We generally believe that the sizes of extracted compounds generally increases with solvent polarity (ethyl acetate < butanol < 95% EtOH < water). However, we cannot say this conclusively, as it is possible for smaller compounds (microbial metabolites and phenolic acids) to be present in water extracts. Concentrations of compounds were different between the cocoa extracts (**Figure 4.4**) and the original cocoa samples (**Figure 4.5**). Between the four extracts, compound levels due to the representative stages of processing were different. In this case, fermentation significantly reduced the amount of ethanol-soluble compounds in both the CEs and original samples. Again, it is important to note the four cocoa samples were not obtained from the same source; therefore, we cannot make absolute conclusions on the effects of fermentation and roasting, only generalized inferences. In the CEs, FL had the highest amount of ethyl acetate- and water-soluble compounds compared to the other four CEs, suggesting fermentation in conjunction with roasting increases both of these classes of compounds (smallest and largest compounds). UL had the highest absorbance of butanol-soluble compounds compared to the other CEs, suggesting that unfermented, roasted cocoa maintains the largest

amount of these compounds. Fermentation significantly reduced the amount of ethanol-soluble compounds in the CEs as UB and UL had higher amounts of these than FB and FL.

As for the original cocoa products, effects of fermentation and roasting on levels of oxidative polymers were different as well. UB and UL had higher amounts of ethanol-soluble compounds than FB and FL in both cases of extract and original sample, suggesting that fermentation decreases the concentrations of these compounds. Fermentation and roasting significantly decreased butanol- and water-soluble compounds in the original cocoa samples (UB > FB = UL = FL for both statistical analyses). There were no significant effects of fermentation or roasting on the concentrations of ethyl acetate-soluble compounds. It is difficult to specify and quantify the exact compounds that are being measured in this assay, but it is possible that these classes could potentially affect, or even improve, cocoa's bioactivity.

All CEs bound and precipitated BSA at doses > 100 μg/mL (**Figure 4.6**). When one-way ANOVA with Tukey's HSD was performed on protein binding, no significant differences were found between samples at concentrations < 100 μg/mL. For concentrations 200 – 2000 μg/mL, UL had a significantly higher absorbance value than the rest on the samples. At 1000 and 2000 μg/mL concentrations, binding ability of the extracts was UL > UB = FL > FB. Therefore it appears as though fermentation reduces and roasting enhances protein-binding abilities since UL, the unfermented, roasted sample, precipitated the most protein. It would be expected that the unfermented samples bind the most protein, as they contained the highest levels of polyphenols. The rationale for testing protein binding of the CEs relates to the sensation of astringency in flavanol-rich products, which is due to the non-specific binding of salivary proteins by flavanols. The perception of astringency increases with flavanol oligomer and polymer concentration, degree of polymerization, and amount of galloyl subunits.[91, 159, 195] Upon examining protein precipitation of 50-2000 ppm solutions of cocoa seed monomers to octamers, Harbertson *et al.*[162] found that turbidity increased with increased DP (monomers and dimers did not precipitate the BSA). When linear regression was performed on our previously-reported mDP data (measured by thiolysis) versus protein binding at 2000 μg/mL, there was a negative correlation (R=0.741) between the two (**Figure 4.9A**), indicating that as the mDP increased, protein binding ability decreased (the opposite of what has been reported), which may be due to the presence of other compounds either in the native cocoa beans or produced by fermentation or roasting that decrease protein binding. Although these observations were made in the present study, it may be

more beneficial to examine specific DP standards versus precipitation to make more accurate conclusions for each DP classification to remove various effects from the many different compounds present in the CEs. Utilizing standards would demonstrate specific effects from one type of compound without any background interference. Conversely, linear regression of total polyphenols measured by the Folin-Ciocalteu assay (mg +C equivalents/g CE) versus protein binding at 2000 μg/mL (**Figure 4.9B**) demonstrated that as concentration of total polyphenols increased, so did protein binding (R=0.892), indicating that a higher concentration of polyphenols results in more effective protein precipitation, which agrees with previous findings. The same was true in the correlation between total flavanols measured by the DMAC assay (mg PCB2 equivalents/g CE) and protein binding at 2000 μg/mL (**Figure 4.9C**), in that increased flavanol concentration improved binding (R=0.429).

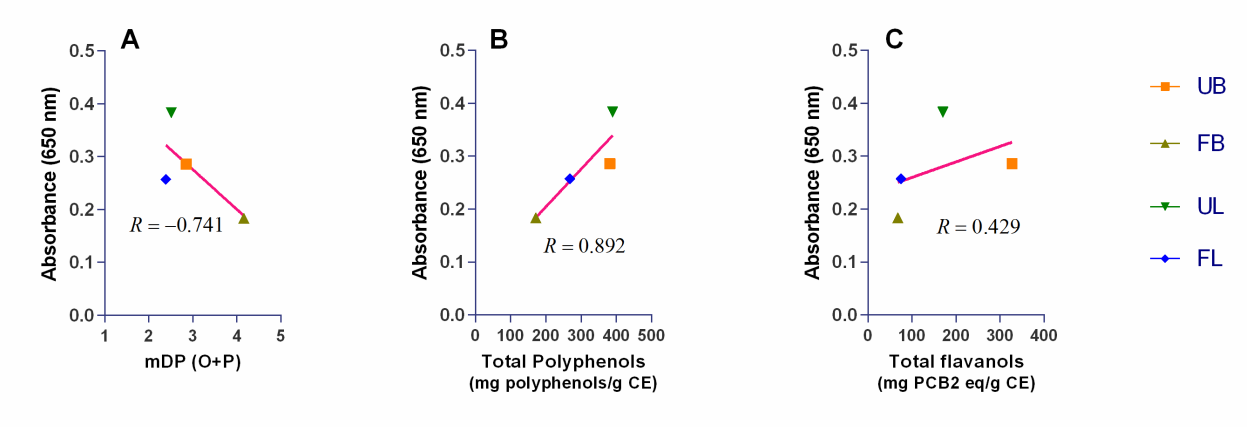


Figure 4.9. Correlations between CE protein binding abilities at 2000 μg/mL and previously reported data for (A) mDP; (B) total polyphenols; and (C) total flavanols

Protein binding can be a highly complex area of research, therefore these methods and observations are very preliminary. Since turbidity is affected greatly by size and number of particles, it is unclear as to how relatable this is to protein binding. Additionally, there could be competition with non-specific binding in the gut, which may influence the effectiveness of this mechanism. For example, proteins the gut would bind and inactivate polyphenols whereas digestion would release these compounds.

Linear regression analyses were performed on IC_{25} values for DPP4 inhibition and our previously reported data for total polyphenols, total flavanols, total monomeric anthocyanins, and mDP in the CEs, as well as correlations with pigment and protein binding data from the current study (**Figure 4.10**) in order to identify properties that predict inhibitory activity. The strongest correlation (R=0.979) was found between protein binding at 2000 μg/mL and IC_{25} values; however, this correlation suggests that as the CEs are more able to bind protein, their DPP4 inhibiting abilities decrease (**Figure 4.10E**). Therefore, protein-binding abilities strongly hindered DPP4 inhibition. Strong correlation (R=0.810) was also found between mDP and IC_{25} (**Figure 4.10D**), suggesting that as mDP of compounds increased, IC_{25} decreased, thus increasing DPP4 inhibition. As for specific classes of compounds, no strong correlations were found between IC_{25} and total flavanols nor anthocyanins. Strong correlation was found between IC_{25} and total polyphenols, butanol- and ethanol-soluble compounds; however, these relationships suggest that as concentrations of these compounds increase, so does IC_{25}, making inhibition less effective. Knowing the exact masses of compounds soluble in these solutions would be useful to understand their sizes and other properties related to bioactivity better. These correlation analyses suggest that increases in mDP of polyphenolic compounds may improve DPP4 inhibition, but that total polyphenols, protein binding abilities, and certain classes of oxidative polymers actually hinder inhibition. It is possible that there may be compounds present in the butanol and ethanol extracts that counter DPP4 inhibition. Therefore, it would be highly beneficial to repeat this study with purified compounds (if possible) to draw conclusions on the effects of these extracts. Again, this draws on the difficulty in isolating and identifying these unknown compounds in order to use as standards.

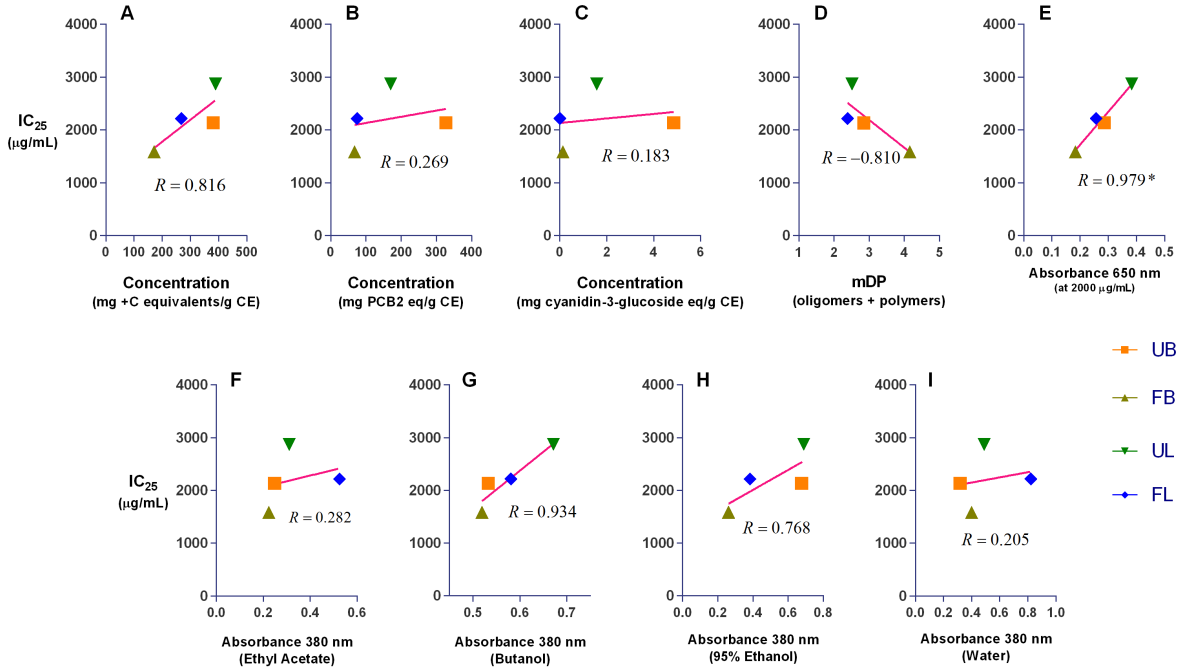


Figure 4.10. Correlation between DPP4 IC_{25} and previously reported data for (A) total polyphenols; (B) total flavanols; (C) total monomeric anthocyanins; (D) mean degree of polymerization; (E) protein binding at 2000 μg/mL; (F) ethyl acetate-soluble compounds; (G) butanol-soluble compounds; (H) 95% ethanol-soluble compounds; and (I) water-soluble compounds. Asterisks (*) beside R values indicate slopes that are significantly non-zero ($p < 0.05$).

Figure 4.11 also shows linear regression analysis of the polymeric pigments in the extracts and protein binding (at 2000 μg/mL) data versus our previously reported digestive enzyme IC_{50}/IC_{25} values in order to determine potential correlations between compound presence and/or protein binding with enzyme inhibition by CEs. In the case of α-glucosidase inhibition, there were moderate to strong correlations between presence of ethyl acetate-, butanol-, and water-soluble compounds and IC_{50} values (**Figure 4.11A, B, and D**, respectively), indicating that as concentrations of these compounds increased, inhibition improved. Studies have reported that α-glucosidase inhibition depends on the presence of smaller, low molecular weight compounds,[16, 136] which may explain the correlations found with ethyl acetate- and butanol-soluble compounds, since these solvents likely extracted smaller compounds. Honda and Hara[172] suggested anti-diabetic effects of tea theaflavins via an intestinal glucosidase mechanism in rats, demonstrating a potential similarity in bioactivity with presence of smaller compounds and α-glucosidase inhibition by CEs.

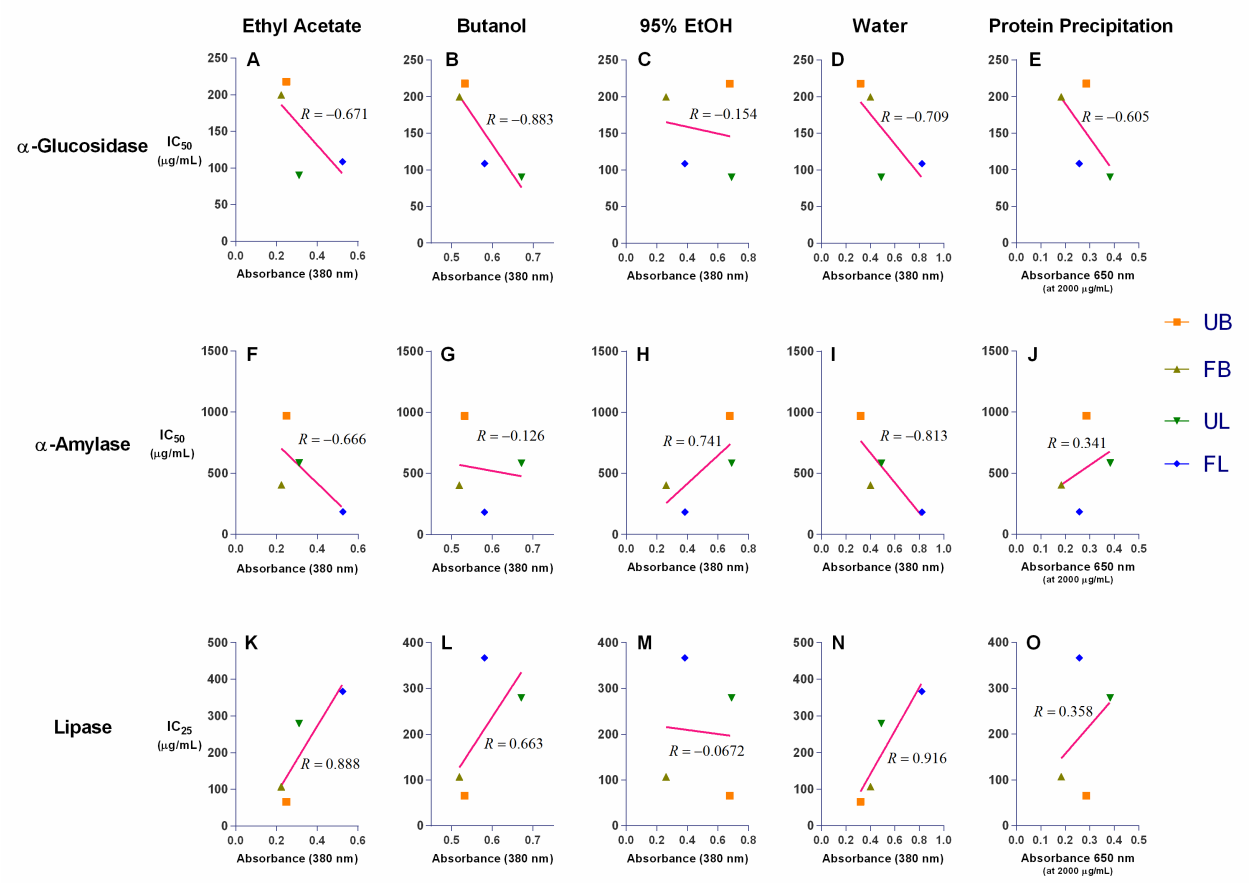


Figure 4.11. Correlation between soluble pigments present in cocoa extracts, protein binding at 2000 ppm and digestive enzyme IC_{50}/IC_{25} values as determined previously. Graphs demonstrate linear regressions between (A) ethyl acetate-soluble compounds and α-glucosidase inhibition; (B) butanol-soluble compounds and α-glucosidase inhibition; (C) ethanol-soluble compounds and α-glucosidase inhibition; (D) water-soluble compounds and α-glucosidase inhibition; (E) protein binding at 2000 ppm and α-glucosidase inhibition; (F) ethyl acetate-soluble compounds and α-amylase inhibition; (G) butanol-soluble compounds and α-amylase inhibition; (H) ethanol-soluble compounds and α-amylase inhibition; (I) water-soluble compounds and α-amylase inhibition; (J) protein binding at 2000 ppm and α-amylase inhibition; (K) ethyl acetate-soluble compounds and lipase inhibition; (L) butanol-soluble compounds and lipase inhibition; (M) ethanol-soluble compounds and lipase inhibition; (N) water-soluble compounds and lipase inhibition; and (O) protein binding at 2000 ppm and lipase inhibition. Strong negative linear regressions indicate correlation between pigment compound/protein binding and enzyme inhibition ability of the CEs.

Similarly, increases in ethyl acetate- and water-soluble compounds (**Figure 4.11F and I**) were moderately correlated with decreases in α-amylase IC_{50} values (improving inhibition). However, increases in ethanol-soluble compounds appeared to hinder amylase inhibition **(Figure 4.11H**). Recently, Cheng *et al*.[196] found that an ethyl acetate fraction of Qingzuhan tea (a dark tea) inhibited α-amylase more effectively than other solvent fractions, including butanol and chloroform. This shows promise for the presence of similar compounds in cocoa to have amylase-inhibiting capabilities as well. Additionally, studies suggest that α-amylase inhibition is improved as DP of compounds increases,[16, 124] which agrees with our strong correlation between inhibition and water-soluble compounds, as we expect these to be the largest.

An important consideration in the exploration of these other compounds is the extraction method used. Our cocoa samples were extracted with a 70:28:2 acetone:water:acetic acid solution, a method that is utilized to optimize extraction of flavanol monomers and procyanidins. While this is the optimal method for flavanol extraction, there still remains other compounds in the extracts besides flavanols that may possess bioacitivity. If the data suggest that other compounds besides flavanols to possess significant bioactivities, one might consider a different extraction method in order to select for oxidative polymers rather than flavanols. We believe that an activity-guided extraction method may be the best approach to find the optimal method. This might include sequential extraction/fractionation of compounds and testing their enzyme inhibition in order to determine which classes of compounds are most bioactive.

Correlations were found with ethyl acetate-, butanol-, and water-soluble compounds with lipase inhibition (**Figure 4.11K, L, and N**). However, in these cases, increases in the concentration of these compounds increased IC_{25} values, indicating that lipase inhibition is decreased as more of these compounds are present. Normally, one would expect inhibition to increase with higher levels of bioactive compounds, therefore it is unclear as to why these hindrances of inhibition were observed.

Since we believe that some of CEs inhibitory activities may be due to non-specific protein binding, we hypothesized that inhibition could be correlated to BSA precipitation solution. Therefore, we ran linear regression analysis on protein binding abilities at 2000 μg/mL versus digestive enzyme IC_{50}/IC_{25} values (**Figure 4.11E, J and O**). In order to conclude that a strong correlation between protein binding and inhibition exists, IC values would need to decrease as protein precipitation absorbance increases. This was only shown in the case of α-

glucosidase, with R=0.605 (**Figure 4.11E**). Therefore, our data suggest that protein-binding abilities of CE is generally not well correlated to its enzyme inhibition abilities, which makes sense since protein binding was well-correlated with total polyphenols and flavanols, which were generally not well-correlated with inhibitory activities. This relationship depends on the concentration of extract and enzyme of interest. Therefore, non-specific protein binding might account for some α-glucosidase inhibition but is likely not the main mechanism by which enzyme inhibition occurs and may be due to more specific mechanisms that we have not yet measured.

In conclusion, this study demonstrated cocoa extracts as inhibitors of DPP4. We then correlated cocoa's inhibition of α-glucosidase, α-amylase, pancreatic lipase, and DPP4 to levels of individual flavanols, protein-binding abilities, and oxidative polymers present in the extracts. Our previous findings were further supported by this study, in that enzyme inhibition by cocoa is not uniformly affected by fermentation and roasting. Very few correlations were found between polyphenol characteristics and bioactivities, which suggests that inhibition is not necessarily dependent on higher concentrations of polyphenols/flavanols. Based on our results, we believe that further research is necessary to identify and specify other bioactive compounds present in cocoa, which may represent a previously uncharacterized pool of dietary bioactives.

Chapter 5

Conclusions and Future Work

Our results from Chapter 3 show promise for cocoa to contribute to prevention of Type-2 diabetes and obesity via inhibition of digestion. Additionally, we believe the study described in Chapter 4 to be highly promising for the exploration of other potentially bioactive components in cocoa beyond the flavanols. However, further research is warranted 1) to explore digestive enzyme inhibition mechanisms and 2) to isolate and identify other compounds and draw correlations between these compounds and cocoa bioactivities.

To overcome limitations of this research, cocoa samples for future studies should be from the same source and processed under the same conditions. Beans should be fermented and/or roasted, liquors should be produced from these beans, and cocoa powder should be produced under laboratory conditions with samples taken at appropriate points in the process. Having more control over processing will allow the resulting products to be compared directly to elucidate processing. The effects of alkalization on cocoa bioactivity also warrant study. Additionally, we used one representative product from each representative class of processing for this preliminary study. Since our data suggest that future studies are warranted, increased sample size (i.e. different products from different sources and processing conditions) for each of the four types of products should be used to facilitate stronger statistical conclusions about correlations between processing, polyphenol composition, protein binding, concentrations of oxidative polymers, and bioactivity. We believe beneficial studies to perform in the near future would include:

- Dry down the pooled solvents utilized for protein-binding to determine the masses of compounds extracted.
- UPLC-MS/MS analysis of precipitated protein pellets in order to identify which compounds bound protein most effectively.
- Other more specific measurements of turbidity including centrifugation, dialysis, fluorescence, conformational change, etc.
- MALDI-TOF or NMR-based analysis of oxidative polymer compounds to characterize their structures and interactions with protein.
- Measurement of redox potential of the ethyl acetate, butanol, ethanol, and water extracts to determine further bioactivities of the different extracts.

- Activity-guided extractions to identify bioactive solvent fractions.

A more long-term research goal that may be warranted includes *in vivo* studies utilizing diets supplemented with extracts from cocoa processed to various degrees and measuring postprandial biomarkers such as enzyme activity, blood glucose levels, GLP-1 and GIP levels, fat excretion, caloric intake/satiety, and weight gain. If cocoa processing could be controlled under a lab setting as described above and samples taken at various stages of the process, different cocoa powders could be formulated into animal diets. This way we know the exact process by which the cocoa is produced and we can make conclusive observations on specific effects of the extracts *in vivo*. One might even fractionate the extracts based on degree of polymerization to delve even further into the effects of both processing and DP on the bioactivities of cocoa.

As demonstrated in this thesis work and research that has been performed previously, there is much promise for the use of cocoa in aiding the prevention and/or amelioration of Type-2 diabetes and obesity. This area of research is extremely exciting as it opens doors for many different mechanisms by which cocoa demonstrates these beneficial bioactivities. The research performed for this thesis not only solidifies some of the previous conclusions that have been made about effects of cocoa processing on polyphenol levels and inhibition of digestive enzymes, but also paves the way for further, more in-depth studies on how processing affects cocoa bioactivity both *in vitro* and *in vivo*. The most impactful findings from this research were those that challenge the central dogmas that have been well accepted for many years. While it is generally believed that bioactivity is proportional to polyphenol concentration, this research has suggested the exact opposite, providing preliminary evidence for a potentially warranted shift of focus to new areas and concepts related to cocoa polyphenol bioactivities.

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Appendices

Appendix A: Cocoa Extraction Protocol

Reagents

1. Cocoa samples
2. Hexane
3. 70:28:2 Acetone:MilliQ Water:Acetic Acid solution

Materials

1. Gloves, goggles
2. 100 mL graduated cylinder
3. 1000 mL, 500 mL, 250 mL beakers
4. Waring laboratory blender, 1 Liter (model #58977-169)
5. Sonicator
6. Hot plate, stir bar
7. 250 mL centrifuge tubes
8. Avanti J-E Centrifuge
9. Large drying plate
10. 500 mL RotoVap flask
11. IKA RV 10 Basic (RotoVap)
12. Freeze dryer
13. Freeze dryer flask

Preparation

1. Measure out ~100 g of cocoa. This will give a yield of ~10 grams of cocoa extract (CE).
2. Pre-freeze 250 mL beaker and freeze dryer flasks for freeze dryer in -20°C freezer.
3. Turn on the RotoVap.

Method

Grinding of Cocoa Samples (if applicable)

1. Place weighed cocoa into plastic weigh boats. Place these in rubber bins.

2. Pour liquid nitrogen over the cocoa and let sit for ~2 min.
3. Pour off excess liquid nitrogen into the rubber bin to evaporate.
4. Place cocoa into the laboratory blender and blend on high for 1 min or until cocoa is ground into a powder-like substance.
5. If necessary, use the mortar and pestle to ensure the sample is completely ground.
6. Record weight of resulting powder.

De-fat Cocoa & Remove Fiber

1. Place measured cocoa and 400 mL of hexane in a 1000 mL beaker.
2. Sonicate the mixture for 10 min, ~22°C.
3. Add a stir bar to the beaker and stir on a hot plate for 5 min.
4. Pour mixture into three 250 mL centrifuge tubes.
5. Centrifuge for 5 min at 5000 x g using the Beckman Coulter Avanti J-E Centrifuge, Rotor 14JA.
6. Discard the supernatant and transfer the cocoa back into the 1000 mL beaker.
7. Repeat steps 1-5.
8. Discard the supernatant and add 10-20 ml hexane in each centrifuge tube and shake well before pouring all the contents to the silver drying plate. Allow the remaining hexane to evaporate under hood. The cocoa should turn from a muddy sludge back into a dry, light brown powder.

Extract Polyphenols

1. Place the de-fatted cocoa in another 1000 mL beaker.
2. Add ~400 mL of 70:28:2 Acetone:MilliQ Water:Acetic Acid solution.
3. Sonicate the mixture for 10 min, ~22°C.
4. Add a stir bar to the beaker and stir on a hot plate for 5 min.
5. Pour the mixture into three 250 mL centrifuge tubes.
6. Centrifuge for 5 min at 5000 x g using the Beckman Coulter Avanti J-E Centrifuge.
7. Pour the supernatant into a clean 2000 mL bottle, avoiding the transfer of any solid particles.
8. Scrape the cocoa back into the 1000 mL beaker.

9. Repeat steps 2-8 at least 2 more times, until the resulting supernatant is essentially colorless.
10. Allow the acetone to evaporate out of the solution. This may need to be done in batches; RotaVap no more that ~200 mL at one time. If the solution stops boiling, increase the vacuum. If the vacuum cannot be turned up anymore and the solution is not boiling, then it is done.
11. Turn on the freeze dryer to pre-cool.
12. Pour the CE into pre-cooled 250 mL beakers and then place beaker in pre-cooled freeze drying flasks. The liquid should make only a thin layer on the bottom. Use multiple beakers/flasks if necessary.
13. Place the freeze drying beakers/flasks in the -80°C freezer for 20 minutes, or until the CE freezes solid.
14. Place frozen freeze drying flasks with beaker holding the CE onto the freeze dryer with a bin of dry ice underneath flask. Surround/cover the flask as much as possible with dry ice to assure CE stays frozen for as long as possible.
15. Freeze dry the cocoa CE for at least 2 days, until all water has been drawn off and the remaining extract is completely dry (may take more than 2 d).
16. After freeze drying:
    a. Crush the CE into a powder with a spatula.
    b. Measure and record the yield.
    c. Transfer the CE to a labeled 50 mL centrifuge tube and store in the -80°C freezer

Appendix B: Folin-Ciocalteu Protocol

Reagents

1. 40% EtOH
2. 0.2 N Folin reagent
3. 7.5% w/v sodium carbonate in MilliQ water
4. Cocoa extracts
5. External standard (example: (+)-catechin, gallic acid)

Materials

1. 15 mL centrifuge tubes
2. Volumetric flasks
3. Vortex
4. Sonicator
5. 5 mL pipette with tips
6. 1000 µL pipette with tips
7. 300 µL pipette with tips
8. 96 well plate
9. BioTek plate reader (Hulver lab)

Sample Preparation

1. Dilute standard solutions (0.01 – 1.0 mg/mL) with 40% EtOH to prepare for standard curve
    a. 40% EtOH is used as blank (0 mg/mL)
2. Prepare 0.2 mg/mL cocoa extract solutions (*n*=4) in 40% EtOH
    a. Vortex and sonicate to dissolve completely

Overall Protocol

1. In 15 mL tubes, combine 100 µL diluted solution (cocoa extract or standard) with 900 µL MQ water (*n*=4 for each cocoa sample; *n*=2 for standards)
2. Add 2.5 mL of 0.2 N Folin-Ciocalteau reagent to each tube
3. Add 2 mL of 7.5% w/v sodium carbonate solution to each tube
4. Vortex for ~15 sec
5. Allow solutions to sit at room temperature for 2 h
6. Deposit 250 µL of each solution into a 96 well plate
7. Read absorbance at 765 nm using plate reader

Appendix C: 4-Dimethylaminocinnamaldehyde (DMAC) Protocol for Total Flavanols

Reagents

1. DMAC Solution (each full well plate requires 24 mL)
    a. Combine 3.0 mL stock HCl and 27 mL EtOH
    b. Place in 4C refrigerator for 15 min
    c. Add 0.03 g DMAC to the solution and mix thoroughly
    d. After use, DMAC solution is good for up to a week if stored in the dark (store in 4C refrigerator)
2. 1:1 ethanol:water (optional: see sample preparation step 1A)
3. Cocoa extracts
4. External standard (procyanidin B2)

Materials

1. 15 mL centrifuge tubes
2. 300 μL pipette with tips
3. Multichannel pipette with tips and solution reservoirs
4. 96 well plate
5. BioTek plate reader (Hulver lab)

Sample Preparation

1. Preparation of standards for standard curve
    a. *Check with Dr. Neilson to see if there is procyanidin B2 available in the lab (it may already be dissolved in solution)
        i. If there is PCB2 in solution, dilute that solution to final concentrations of 100, 50, 10, and 1 ppm with EtOH
        ii. If we have procyanidin B2 that is NOT already in solution, prepare the standards as follows:
            1. Combine 5 mg procyanidin B2 with 50 mL 1:1 ethanol:water to make a 100 ppm solution
            2. Dilute with EtOH to concentrations of 50, 10, and 1 ppm
    b. EtOH will be used as the blank (0 ppm)
2. Prepare 100 ppm solutions of cocoa extract in EtOH (*n*=4; 10 mg CE diluted to 100 mL with EtOH)

Overall Protocol

1. In a 96 well plate, add 50 μL of either EtOH blank (0 ppm), standard solution (1, 10, 50, 100 ppm), or cocoa extract solution (100 ppm) to the wells
    - Standards should be done in duplicate; cocoa samples *n*=4
2. Add 250 μL DMAC solution to each well
3. Read absorbance at 640 nm using plate reader

Appendix D: Anthocyanin Content Determination

Reagents

1. 0.025 M potassium chloride buffer (pH=1.0)
    a. "Weigh 1.86 g KCl into a beaker and add distilled water to ca 980 mL. Measure the pH, and adjust pH to 1.0 (±0.05) with HCl (ca 6.3 mL). Transfer to a 1 L volumetric flask, and dilute to volume with distilled water" (Lee *et al* 2005)
2. 0.4 M sodium acetate buffer (pH=4.5)
    a. "Weigh 32.81 g sodium acetate in a beaker, and add distilled water to ca 960 mL. Measure the pH, and adjust pH to 4.5 (±0.05) with HCl (ca 20 mL). Transfer to a 1 L volumetric flask, and dilute to volume with distilled water" (Lee *et al* 2005)

Materials

1. Beakers
2. pH meter
3. Volumetric flasks
4. Pipettes with tips
5. 96-well plate
6. Spectrophotometer (plate reader in Hulver lab)

Overall Protocol

1. Dissolve 120 mg* cocoa extracts (n=4) in 1 mL DMSO
    a. Previously determined that the maximum volume of DMSO that can be added to the buffers without changing the pH is about 2% of the total volume
    b. *When testing samples, adjust concentration of sample in DMSO according to its absorbance reading at 520 nm (must be between 0.2 and 1.4)
2. In separate 5 mL volumetric flasks, dilute 0.1 mL of the extract solution to 5 mL with the potassium chloride buffer (pH=1.0) and the sodium acetate buffer (pH=4.5)
3. Transfer 200 µL of each solution into a 96-well plate
4. Read the plate at 520 and 700 nm (read within 20-50 minutes of preparing the solutions)
    a. **Be sure to select "pathlength correction" when setting up the plate reader method. This does multiple wavelength readings to correct the data to a 1 cm pathlength (see info below on Biotek's website)
        i. http://www.biotek.com/assets/tech_resources/DNA_Quant_Tech_Note.pdf

Calculations:

Calculate anthocyanin pigment concentration, expressed as cyanidin-3-glucoside equivalents, as follows:

$$\text{Anthocyanin pigment (cyanidin-3-glucoside equivalents, mg/L)} = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times 1}$$

where $A = (A_{520nm} - A_{700nm})\text{pH } 1.0 - (A_{520nm} - A_{700nm})\text{pH } 4.5$; MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside (cyd-3-glu); DF = dilution factor established in **D**; l = pathlength in cm; ε = 26 900 molar extinction coefficient, in $L \times mol^{-1} \times cm^{-1}$, for cyd-3-glu; and 10^3 = factor for conversion from g to mg.

Figure 1. Anthocyanin calculation from Lee et al. 2005 **NOTE: The DF in this calculation is accounting for the dilution of CE in DMSO with buffer. Therefore, this equation gives you mg anthocyanins/L cocoa extract in DMSO solution. Then use that to back calculate to anthocyanins in CE

Appendix F: Thiolysis Protocol

Reagents (make in advance):

1. 3.3% HCl in water (8.87 mL HCl, dilute to 100 mL)
2. 5% benzyl mercaptan in MeOH
    a. NOTE: due to the safety hazards of this reagent, prepare under fume hood with proper personal protective equipment
3. 95:5 0.1% formic acid in water (phase A):0.1% formic acid in ACN (phase B)

Materials:

1. Gloves, safety glasses, facemask
2. 50-100 μL micropipette and tips
3. Microcentrifuge tubes with screw caps
4. Parafilm
5. HPLC vials and caps
6. Vortex

Preparation (day of experiment):

1. Heat water on hot plate to 90°C under fume hood
2. Allow cocoa extract to defrost to room temperature
3. Prepare ice bath for microcentrifuge tubes

Overall Method:

1. Dilute cocoa extract to 0.5 mg/mL in MeOH (*n*=4; 20 mg CE in 40 mL MeOH; sonicate to dissolve)
2. Prepare supplies under fume hood to carry out remainder of procedure
3. To prepare a control (unthiolyzed) sample, in a microcentrifuge tube, combine (4 reps):
    a. 50 μL diluted cocoa extract sample
    b. 150 μL MeOH
4. To prepare the thiolyzed samples, in a microcentrifuge tube, combine (4 reps):
    a. 50 μL diluted cocoa extract sample
    b. 50 μL 3.3% HCl reagent
    c. 100 μL benzyl mercaptan reagent
5. Parafilm the lids of the tubes tightly
6. Place the thiolyzed tubes in the 90C water bath for 5 min
7. Remove samples from water and place on ice for 5 min
8. To prepare samples for LC/MS:
    a. Transfer 100 μL from the centrifuge tube to an HPLC vial
    b. Add 900 μL of 95:5 Phase A/Phase B
    c. Vortex gently to mix
9. Carry vials down the hall in a Tupperware container and analyze using LC/MS method

*Prepare one CE sample at a time (8 tubes) and run those on the LC. While those are being analyzed (8 samples = 88 minutes to analyze), prepare the next sample/next set of 8 tubes

Appendix G: α-Glucosidase Inhibition Protocol

Materials

1. Gloves
2. pH meter
3. Cocoa extracts and Acarbose
4. 1.5 mL microfuge tubes
5. Vortex
6. Graduated cylinders and beakers
7. Pipettes with tips (10 - 1000 μL)
8. Multichannel pipettes with solution reservoirs
9. 96 well plates
10. Plate covers
11. BioTek plate reader (Hulver lab)

Reagents to Make in Advance

- 0.1 M phosphate buffer (pH = 6.9)
    - Combine:
        - 1000 mL MQ water
        - 8.05 g sodium phosphate, monobasic, anhydrous (ACID)
        - 4.67 g sodium phosphate, dibasic, anhydrous (BASE)
    - Use pH probe to check that it is 6.9
        - If not, use phosphoric acid (lower pH) or NaOH (raise pH) to adjust

Reagents to Make on the Day of the Experiment

1. Inhibitors (Acarbose standard or cocoa extract):
    a. Stock solutions in dimethylsulfoxide (DMSO):
        i. Combine 20 mg inhibitor with 250 μL DMSO (80000 μg/mL)
        ii. Perform serial dilutions with DMSO to prepare solutions 40000 – 4 μg/mL
    b. Working sample solutions (*n*=6 for each concentration)
        i. Dilute each of the stock solutions 10 fold (combine 10 μL stock solution with 90 μL MQ water) to use as the working solutions (concentrations range from 0.4 – 8000 μg/mL)
        ii. Blank (0 μg/mL) = 90 μL water + 10 μL DMSO

1. 1 mM *p*-nitrophenyl α-D-glucopyranoside (*p*NPG) in 0.1 M phosphate buffer substrate solution
    - Weigh out 30 mg (0.030 g) pNPG (Sigma Aldrich; stored in -20C freezer) and to dilute to 100 mL with phosphate buffer

2. α-glucosidase solution (Sigma Aldrich; 23 U/mg solid; 100 U per bottle; stored in -20C freezer)
    - Combine 100 units of enzyme with 100 mL phosphate buffer and mix thoroughly

- Final concentration = 1 U/mL in 0.1 M phosphate buffer (pH = 6.9)

Overall Protocol

1. In 96 well plate (*n*=6), combine:
    a. 50 µL inhibitor working solution (0 - 8000 µg/mL working solutions; 0 - 2000 µg/mL in reaction)
    b. 100 μL enzyme solution (0.5 U/mL in reaction)
        i. For 0 activity blank (no enzyme, no inhibitor) sample, add 0.1 M phosphate buffer instead
2. Incubate at 25C (room temperature) for 10 min
3. Add 50 µL *p*NPG solution (1.25 mM in reaction)
4. Read absorbance at 405 nm
5. Incubate at 25C for 5 min (leave in plate reader for 5 min)
6. Read absorbance again at 405 nm

Calculations:

% α-Glucosidase Activity will be calculated using the following equation:

$$\%\ \alpha - Glucosidase\ Activity = \left(\frac{\Delta A_{sample}}{\Delta \bar{A}_{blank}}\right) x\ 100$$

Where:

ΔA_{sample} = the change in the individual absorbance value of the product of the inhibitor, substrate, and enzyme at each inhibitor dose before and after incubation

$\Delta \bar{A}_{blank}$ = the average change in absorbance of the blank (0 µg/mL) before and after incubation

Appendix H: α-Amylase Inhibition Protocol

Overview: We are reacting pancreatic alpha-amylase with a substrate, Red Starch, in the presence of an inhibitor (Acarbose or cocoa extract). During incubation, the enzyme works to break down this starch into smaller sugars. We add ethanol to stop the reaction and to precipitate out any undigested starch. The starch itself is insoluble in ethanol, whereas the sugars released as a result of digestion are soluble in ethanol. Centrifugation separates remaining starch from the sugars in the solution. This solution containing the sugars released during digestion is then read on the plate reader at 510 nm, which is correlated to alpha-amylase activity. As the red color fades, alpha-amylase activity decreases. The role of the inhibitors is to delay the activity of alpha-amylase, thus delaying the digestion of the starch, which can be related to the effects of these inhibitors on postprandial blood glucose levels in the body.

Reagents to make in advance:

1. 95% ethanol solution
2. 20 mM Phosphate buffer (pH=6.9) containing 6.7 mM sodium chloride
    a. Combine:
        i. 1.61 g sodium phosphate, monobasic, anhydrous (ACID)
        ii. 0.93 g sodium phosphate, dibasic, anhydrous (BASE)
        iii. Dilute to 1000 mL with MQ water
    b. Use pH probe to check that it is 6.9
        i. If not, use phosphoric acid (lower pH) or NaOH (raise pH) to adjust
    c. Add 0.39 g NaCl and stir to mix completely
3. Red Starch stock solution (20 mg/mL)
    a. First make 0.5 M KCl
        i. Combine 1.86 g KCl in 50 mL MQ water
    b. Then add 1.0 g red starch (Sigma Aldrich) to the 50 mL 0.5 M KCl
    c. Stir at 60C until dissolved

Preparation (day of experiment):

- Turn on water bath to 37C

Prepare Reagents (day of experiment):

1. Amylase solution (our Amylase is 15,000 U/g; Sigma Aldrich; stored in 4C refrigerator)
    a. In a 1.5 mL tube: combine 1.25 mL phosphate buffer then 0.25 g (250 mg) amylase from porcine pancreas
        i. This yields a 3000 U/mL solution
    b. Mix contents by vortexing and pipetting solution up and down
    c. Centrifuge at 1000 x g for 10 min
    d. Dilute 100 µL of the supernatant to 10 mL (100 fold) with phosphate buffer
        i. This yields a 30 U/mL solution

2. Inhibitors (Acarbose or Cocoa Extract)
    a. Stock solutions in DMSO (vortex/sonicate to mix as needed)
        i. Combine 70 mg inhibitor with 250 µL DMSO (280,000 µg/mL)
        ii. Perform serial dilutions with DMSO to prepare solutions 140,000 – 14 µg/mL
    b. Working sample solutions (*n*=6) (10 fold dilution)
        i. Dilute each of the stock solutions 10 fold (combine 10 µL stock solution with 90 µL MQ water) to use as the working solutions (concentrations range from 1.4 – 28,000 µg/mL)
        ii. Blank (0 µg/mL) = 90 µL water + 10 µL DMSO

Overall Procedure:

1. Pre incubate red starch stock solution and amylase solution in 37°C waterbath for 5 min
2. In 96 well plate, combine:
    a. 25 µL red starch solution (7 mg/mL in reaction)
    b. 20 µL MQ water
    c. 5 µL inhibitor solution (0 - 2000 µg/mL in reaction)
    d. 20 µL amylase solution (8.5 U/mL in reaction)
        i. FOR 0 ACTIVITY BLANK: ADD BUFFER INSTEAD
3. Cover plate and place on plate shaker to mix contents
4. Incubate covered plates in 37°C waterbath for 20 min, oscillating slowly (40 rpm)
5. Add 200 µL 95% ethanol to end reaction
6. Cover plate and place on plate shaker to mix contents
7. Let samples sit until they reach room temp (~5 min)
8. Centrifuge plates at 6,000 x g, 30 min, 4°C
9. Transfer 200 µL of the supernatants to a new 96 well plate
10. Read absorbance at 510 nm

Calculations:

% amylase activity will be calculated using the following equation:

$$\%\,\alpha - Amylase\ Activity = \left(\frac{A_{I,S,E}}{\bar{A}_{S,E}}\right) x\ 100$$

Where:

$A_{I,S,E}$ = the individual absorbance value of the product of the inhibitor, substrate, and enzyme at each inhibitor dose

$\bar{A}_{S,E}$ = the average absorbance value of the reaction with substrate and enzyme without inhibitor (0 μg/mL)

Appendix I: Pancreatic Lipase Inhibition Protocol

Materials

1. Gloves
2. pH meter
3. 1000 mL beaker
4. Hot plate
5. Cocoa extracts and Orlistat
6. 1.5 mL microfuge tubes
7. Vortex
8. Microfuge
9. Pipettes with tips (10 - 1000 µL)
10. Multichannel pipettes with solution reservoirs
11. 96 well plates
12. Plate covers
13. Oscillating waterbath
14. BioTek plate reader (Hulver lab)

Reagents to be Made in Advance:

1. 100 mM Tris buffer (pH 8.2)
    a. Dissolve 15.76 g Trizma-HCl in 1000 mL MQ water
    b. Adjust to pH 8.2 with NaOH
2. 5 mM Sodium acetate (pH 5.0) containing 1% Triton X-100
    a. In a 500 mL volumetric flask, combine:
        i. 0.2051 g sodium acetate
        ii. 5 mL Triton X-100
    b. Bring to volume with MQ water
    c. Check pH and adjust with acetic acid

Before starting (day of experiment):

- Turn on water bath to 37C
- Boil water in 1000 mL beaker on hot plate

Reagents (Prepare day of experiment):

1. Inhibitors (Orlistat standard or cocoa extract):
    - Stock solutions in Dimethylsulfoxide (DMSO)
        - Combine 105 mg inhibitor with 250 µL DMSO (420000 µg/mL)
        - Perform serial dilutions with DMSO to prepare solutions 210000 – 21 µg/mL
    - Working sample solutions (*n*=6) (10 fold dilution)
        - Dilute each of the stock solutions 10 fold (combine 10 µL stock solution with 90 µL MQ water) to use as the working solutions (concentrations range from 2.1 – 42000 µg/mL)
        - Blank (0 µg/mL) = 90 µL water + 10 µL DMSO

2. 10 mg/mL Lipase in DI water
    - Measure out 10 mg pancreatic lipase (Sigma Aldrich; in 4C refrigerator), place into 1.5 mL tube containing 1 mL MQ water
    - Prepare at least three tubes of this per sample
    - Centrifuge at 13,300 rpm for 5 min and use supernatant as working solution

3. Substrate solution:
    - 0.08% w/v 4-nitrophenyl dodecanoate (Sigma Aldrich; in 4C refrigerator) in 5 mM sodium acetate (pH 5.0) containing 1% Triton X-100
        - If doing 1 sample per day:
            - Make 20 mL
                - 0.016 g pNP laurate in 20 mL 5 mM sodium acetate (pH 5.0) containing 1% Triton X-100
        - If doing 2 samples per day:
            - Make 40 mL
                - 0.032 g pNP laurate in 40 mL 5 mM sodium acetate (pH 5.0) containing 1% Triton X-100
        - Heat in boiling water for 1-2 min to aid in dissolution then cool to room temperature

Overall Protocol:

1. In 96 well plate, combine:
    a. 80 μL assay buffer
    b. 30 μL lipase solution (1.429 mg/mL in reaction)
        i. For 0 activity standards (no enzyme, no inhibitor), add water instead
    c. 10 μL diluted cocoa extract solution (n=6; 0 – 42000 μg/mL working solutions; 0 – 2000 μg/mL in reaction)
    d. 90 μL substrate solution (0.0343% pNP in reaction)
2. To obtain the substrate and inhibitor (no enzyme) data, in a separate 96 well plate, combine:
    a. 80 μL assay buffer
    b. 30 μL water
    c. 10 μL diluted cocoa extract solution (n=6; 2.1 – 42000 μg/mL working solutions; 0.1 – 2000 μg/mL in reaction)
    d. 90 μL substrate solution (0.0343% pNP laurate in reaction)
3. Cover plates and incubate at 37C for 2 hours in water bath, oscillating at 40 rpm
4. Dry off plates and read absorbance at 400 nm using plate reader

Calculations:

% lipase activity will be calculated using the following equation:

$$\%\ Lipase\ Activity = \frac{(A_{I,S,E} - \bar{A}_{I,S})}{(\bar{A}_{S,E} - \bar{A}_{S})}\ x\ 100$$

Where:

$A_{I,S,E}$ = the individual absorbance value of the product of the inhibitor, substrate, and enzyme at each inhibitor dose

$\bar{A}_{I,S}$ = the average absorbance value of the inhibitor and substrate (no enzyme) reaction

$\bar{A}_{S,E}$ = the average absorbance value of the reaction with substrate and enzyme without inhibitor (0 μg/mL)

$\bar{A}_{S}$ = the average absorbance value of the substrate without enzyme and inhibitor

Appendix J: Dipeptidyl Peptidase-IV (DPP4) Inhibition Protocol

Reagents (make in advance):

1. 100 mM Tris buffer (pH = 8.0)
    a. Dissolve 15.76 g Trizma-HCl in 1000 mL MQ water
    b. Adjust to pH 8 with NaOH
2. 3% acetic acid

Before starting (day of experiment):

- Turn on water bath to 37C

Reagents (make day of experiment):

1. Inhibitors (Ile-Pro-Ile standard; 10 mg):
    a. Stock solutions in DMSO
        i. Combine 10 mg standard with 125 µL DMSO (yields an 80000 µg/mL solution)
        ii. Perform serial dilutions with DMSO to prepare solutions 40000 – 4 µg/mL
    a. Working sample solutions (*n*=6 for each concentration)
        i. Dilute each of the stock solutions 10 fold (combine 10 µL stock solution with 90 µL MQ water) to use as the working solutions (concentrations range from 0.4 – 8000 µg/mL)
        ii. Blank (0 µg/mL) = 90 µL MQ water + 10 µL DMSO
2. Inhibitors (cocoa extract):
    a. Stock solutions in DMSO
        i. Combine 20 mg CE with 250 µL DMSO (yields an 80000 µg/mL solution)
        ii. Perform serial dilutions with DMSO to prepare solutions 40000 – 4 µg/mL
    b. Working sample solutions (*n*=6 for each concentration)
        i. Dilute each of the stock solutions 10 fold (combine 10 µL stock solution with 90 µL MQ water) to use as the working solutions (concentrations range from 0.4 – 8000 µg/mL)
        ii. Blank (0 µg/mL) = 90 µL MQ water + 10 µL DMSO

3. DPP4 working solution (vials stored in -80C; each vial is 25 mU and 1.7 mU/uL, therefore each vial contains 14.71 uL enzyme solution)
    a. Working solution needs to be ~0.002 units/mL in buffer (2 mU/mL buffer)
    b. To make enough enzyme for one sample (3.3 mL per sample):
        i. Dilute 3.88 uL from vial to 3.3 mL with 100 mM Tris buffer (pH = 8.0)
    c. To use the rest of that vial (10.83 uL remaining; enough for two more samples):
        i. Dilute 10.83 uL to 9.2 mL with buffer
    d. To use entirety of one vial (enough for three samples):
        i. Dilute vial contents (14.71 uL) to 12.5 mL with buffer
    e. NOTE: Do not dilute vial contents until ready to use; only dilute as much enzyme as needed at the time; avoid thawing and re-freezing of the enzyme

4. 1 mM GP-pNA substrate solution (MW = 328.75 g/mol)
    a. Combine 0.0164 g GP-pna with 50 mL water
    b. Each sample (2 plates) needs ~6.6 mL

Overall Procedure:

1. In 96 well plate, combine:
    a. 50 μL inhibitor solution (0-2000 μg/mL in reaction)
    b. 50 μL 2 mU/mL DPP4 in buffer solution
2. In a separate 96 well plate, combine:
    a. 50 μL inhibitor solution (0-2000 μg/mL in reaction)
    b. 50 μL 100 mM Tris buffer solution (pH = 8)
3. Cover plates and incubate in 37C waterbath for 10 min
4. Add 50 μL 1 mM GP-pNA to each well
5. Add 50 μL 100 mM Tris buffer (pH=8) to each well
6. Cover plates and incubate at 37C for 60 min, oscillating slowly
7. Add 50 μL 3% acetic acid to each well to end the reaction
8. Read absorbance at 405 nm

Calculation:

% DPP4 activity will be calculated using the following equation:

$$\%\ DPP4\ Activity = \frac{(A_{I,S,E} - \bar{A}_{I,S})}{(\bar{A}_{S,E} - \bar{A}_{S})}\ x\ 100$$

Where:

$A_{I,S,E}$ = the individual absorbance value of the product of the inhibitor, substrate, and enzyme at each inhibitor dose

$\bar{A}_{I,S}$ = the average absorbance value of the inhibitor and substrate (no enzyme) reaction

*this will be a different average value for each dose of inhibitor

$\bar{A}_{S,E}$ = the average absorbance value of the reaction with substrate and enzyme (no inhibitor; this is the same as the average of the 0 µg/mL blank values)

$\bar{A}_{S}$ = the average absorbance value of the substrate (no enzyme, no inhibitor) (0 activity standard)

The numerator represents inhibited DPP4 activity, whereas the denominator represents uninhibited DPP4 activity

Appendix K: Protein Precipitation Protocol

Reagents to make in advance:

1. Citrate/phosphate buffer (pH 3.8)
    a. Combine:
        i. 322.5 mL 0.1 M citric acid
            1. 21.01 g citric acid monohydrate/1000 mL dd H2O
        ii. 177.5 mL 0.2 M sodium phosphate
            1. 28.40 g sodium phosphate (MW=141.98)/1000 mL dd H2O
    b. Mix thoroughly and check that pH=3.8 (adjust with HCl or NaOH if needed)

Reagents to make day of:

1. 2 mg/mL BSA in buffer
2. Diluted cocoa extract solutions:
    c. Stock solutions in dimethylsulfoxide (DMSO)
        i. Combine 20 mg inhibitor with 500 μL DMSO (yields a 40000 μg/mL solution)
        ii. Perform serial dilutions with DMSO to prepare solutions 20000 – 2 μg/mL
    d. Working sample solutions (*n*=4 for each concentration)
        i. Dilute each of the stock solutions 10 fold (combine 100 μL stock solution with 900 μL citrate/phosphate buffer) to use as the working solutions (concentrations range from 0.2 – 4000 μg/mL)
        ii. Blank (0 μg/mL) = 900 μL citrate/phosphate buffer + 100 μL DMSO

Overall Procedure:

1. In a 96-well plate, combine:
    a. 100 μL 2 mg/mL BSA in buffer solution
    b. 100 μL working CE reaction (0-2000 μL/mL in reaction; *n*=4)
2. In separate wells of the same well plate, combine:
    a. 100 μL citrate/phosphate buffer (pH=3.8)
    b. 100 μL working CE reaction (0-2000 μL/mL in solution; *n*=4)
3. Cover plate and place on plate shaker to mix contents
4. Read absorbance at 650 nm

Appendix L: Polymeric Pigment Method

Reagents

1. 95% EtOH
2. Butyl alcohol
3. Ethyl acetate

Overall Protocol (Cocoa Extracts)

1. Measure out 50 mg (*n*=4) CE and place in individual 2 mL tubes
2. Add 200 μL ethyl acetate to each tube
3. Vortex and sonicate to mix
4. Centrifuge at 17,000 x g, 5 min
5. Pull off supernatants and store in new tubes
6. Repeat extraction with ethyl acetate 2 more times, pooling each individual samples' supernatants
7. Using same CE pellet, repeat steps 2-6 with butanol, 95% EtOH, and dd water in a sequential order
8. Dilute the water extraction supernatants 10x with water and EtOH supernatants 5x with 95% EtOH so that they are within the absorbance range
9. Transfer 250 μL of the pooled supernatants to a 96-well plate
10. Read absorbance at 380 nm

Overall Protocol (Original Samples)

1. Measure out 1 g of each product, freeze in liquid nitrogen, and grind (use mortar and pestle or hammer with weigh boats)
2. Divide each sample into four tubes containing 250 mg of the sample and add 1 mL ethyl acetate to each tube so that each sample has *n*=4 replicates (use 15 mL tubes)
3. Polytron the samples to further grind up samples and mix into the solvent
4. Transfer contents to individual 2 mL centrifuge tubes (*n*=4 tubes for each cocoa sample; each containing roughly 250 mg cocoa and 1 mL ethyl acetate)
5. Centrifuge tubes at 17,000 x g, 5 min
6. Pull off supernatants and store in new tubes (use 15 mL tubes)
7. Repeat extraction with ethyl acetate 2 more times (adding 1 mL solvent to each tube), pooling each individual samples' supernatants
    a. Instead of polytron, vortex and sonicate to mix
8. Using same CE pellet, repeat extractions with butanol, 95% EtOH, and dd water in a sequential order
9. Dilute the water extraction supernatants 2x with water and EtOH supernatants 2x with 95% EtOH so that they are within the absorbance range
10. Transfer 250 μL of the pooled supernatants to a 96-well plate
11. Read absorbance at 380 nm

Notes:

- Procedure adapted from Wang et al 2011 and Zou et al 2014
- Ethyl acetate is known to draw out smaller compounds → roughly correlated to theaflavins
- Zou et al says butanol→thearubigins and 95% →theabrownins; Wang et al says butanol → theabrownins
    - We don't know exactly which reagent corresponds to each of the larger compounds, but we are using this as a rough measurement of the compounds

Appendix M: Experimental Biology 2016 Poster Abstract

Fermentation and roasting of cocoa (*Theobroma cacao*) decrease levels of flavan-3-ols, which may aid in prevention of obesity and type-2 diabetes. Polyphenolic profiles, protein binding abilities, presence of compounds termed "oxidative polymers", and abilities to inhibit α-glucosidase, pancreatic α-amylase, pancreatic lipase, and dipeptidyl peptidase-IV (DPP4)*in vitro* were measured and compared between unfermented bean (UB), fermented bean (FB), unfermented liquor (UL), and fermented liquor (FL) cocoa extracts. Overall, there was a significant decrease ($p<0.05$) in total polyphenols, flavanols, and anthocyanins between the two sets of unfermented and fermented cocoa samples. HPLC and thiolysis demonstrated a decrease in catechins between unfermented and fermented samples as well as an increase in average flavanol degree of polymerization (mDP) from UB to FB. All cocoa extracts effectively inhibited α-glucosidase (UL with the lowest IC_{50} = 90.0 μg/mL) and moderately inhibited α-amylase (lowest IC_{50}=183 μg/mL for FL), lipase (lowest IC_{25}=65.5 μg/mL for FB), and DPP4 (lowest IC_{25}=1585 μg/mL for FB) in dose-dependent manners. Inhibition of each enzyme was affected differently by fermentation and roasting of the samples (both processes improved α-amylase inhibition). Protein binding abilities at high concentrations were UL > UB = FL > FB, suggesting that roasting enhanced binding. Changes in oxidative polymer levels due to the representative stages of processing were different between the CEs. Improved DPP4 inhibition was strongly correlated to increased mDP. Improved α-glucosidase and α-amylase inhibitions were correlated with presence of different classifications of oxidative polymers, suggesting that these compounds could be contributing to the bioactivities observed. Our data suggest that potential enzyme inhibition bioactivities of cocoa are not necessarily negatively affected by the large polyphenol losses that occur during fermentation and roasting and it is possible that non-flavanol bioactive compounds are present in cocoa that contribute to its anti-diabetic and anti-obesity activities.