Effect of Cocoa Proanthocyanidins on Histidine Decarboxylase Activity *In Vitro*

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Introduction

Functional foods are foods that provide a health benefit beyond what would be expected based on nutritive value alone. Fruits and vegetables are good examples of functional foods. Recent studies suggest that increased consumption of fruits and vegetables may play a role in decreasing the risk of cancer, heart disease, stroke, and several other chronic diseases (1, 14). Fruits and vegetables are not the only example of functional foods, however. In fact, research has shown that a wide array of foods and beverages including cereals, legumes, nuts, wine, cider, beer, tea, and cocoa may also inhibit chronic diseases such as cancer and heart disease (6).

Heart disease and cancer are the two leading causes for death in the United States, accounting for over one million deaths annually (1, 15). Because of the prevalence of these diseases, much effort has been focused on identifying the compounds within functional foods that are responsible for the prevention of disease and on clarifying the mechanism(s) by which these compounds act. Through these efforts, polyphenolics, a group of compounds present in many fruits and vegetables as well as tea, cocoa, and nuts, have been isolated as food compounds that show promising results in reducing the risk of cancer and other diseases (8,13).

Polyphenolics are secondary plant metabolites that are formed through the condensation of flavan-3,4-diols. Most consist of the monomeric units of catechin, epicatechin, gallocatechin, or epigallocatechin (4, 6) (see Figure 1). They make up one of the most widespread groups of plant metabolites, and they play a key role in characteristics such as plant pigmentation, growth and reproduction, resistance to pathogens and predators, and protection from plague and pre-harvest seed germination. Polyphenolics are also responsible in part for certain sensory and nutritional qualities of the foods they are found in, including astringency and bitterness, and

they are important in both beneficial and detrimental changes that occur in food due to oxidation (6).

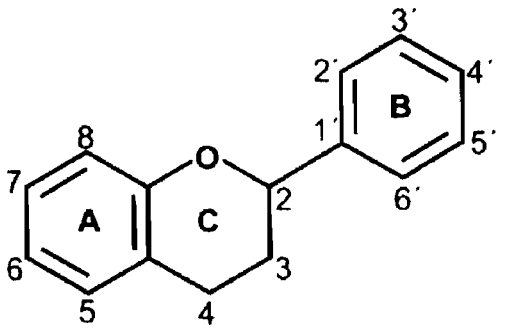

Figure 1. Typical polyphenol structure.

Polyphenolics have several unique structural features that may confer anticancer activity. These features include an o-diphenolic group in the B ring, a 2-3 double bond conjugated with a 4-oxo function, and hydroxyl groups in positions 3 and 5 (2). Because of these features, polyphenolics serve as scavengers of reactive oxygen species, compounds implicated in the pathogenesis of cancer and heart disease (21). However, this scavenging ability is not the only manner in which polyphenolics may prevent cancer. Proanthocyanidins, a subclass of polyphenolics found in fruits and vegetables, have also been shown to inhibit ornithine decarboxylase (ODC), the rate-limiting enzyme in the synthesis of polyamines (9). Polyamines serve as regulators for many vital cell functions including growth, metabolism, differentiation, and proliferation (6, 13). Increased levels of ODC activity are associated with the proliferation of cancer cells, and ODC is thought to play a critical role in the promotion and local proliferation of tumor cells (3, 7, 13, 16).

However, ODC is not the only enzyme necessary for the growth of cancer cells. Histidine decarboxylase (HDC), an enzyme that results in the production of histamine, is catalytically similar to ODC and is also important in the growth of cancer cells. An over

expression of HDC has been found in several different types of tumors including those produced by leukemia, breast cancer, stomach cancer, and lung cancer (13). Histamine itself plays a role in gastric secretions, allergic reactions, inflammation, and smooth muscle contractions. Perhaps more interesting for purposes of this study, it also plays a role in cell proliferation (13). In fact, increased levels of histamine have been found in tissues from animals with tumors. In addition, increased levels of both histamine and HDC activity have been reported during the exponential phase of tumor growth (3). The increase in HDC activity within cancer cells parallels the elevation of ODC activity observed in the development of many cancers (2, 13). In addition, histamine is also involved in the development of heart disease and in the aggravation of both high blood pressure and low blood pressure conditions.

HDC and ODC share similarities other than similar increases in activity during the early stages of cancer development. The most prominent similarity between ODC and HDC is the presence of PEST regions in both enzymes. PEST regions are sequence fragments rich in proline, glutamic acid, serine, and threonine residues within a hydrophobic fragment surrounded by cationic amino acids. PEST regions can act as signals that provide degradation mechanisms for proteins important in cell metabolism (8,9). Also, because PEST regions are rich in proline, they may bind to proanthocyanidins. Pascale *et al.* reported that proline-rich proteins precipitated proanthocyanidins from grapes and wine, and this same trend has been observed with proanthocyanidins from other foods as well (17). Similarities also exist between the cofactors of the two enzymes as well as the chemical structures of their respective substrates and inhibitors. For example, ODC and HDC are both inhibited by the alkyl-fluoro derivatives of their substrates. For ODC, this inhibitor is α-difluoromethyl ornithine (DFMO), and for histidine it is monofluoromethyl histidine (MFMH). (see Figure 2). Because of these

similarities, Sanchez-Jimenez *et al.* have postulated that similarities also exist between the conformations of the catalytic centers of ODC and HDC (20).

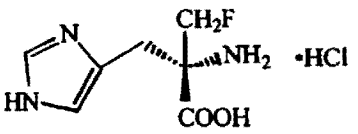

Figure 2. Monofluoromethylhistidine hydrochloride

Because of the similarities between ODC and HDC, proanthocyanidins may likely inhibit the activity of HDC as well as ODC. However, the effects of proanthocyanidins on HDC activity have not been investigated. This study tested the hypothesis that proanthocyanidins inhibit HDC to a similar extent as ODC. As mentioned earlier, proanthocyanidins exist in a variety of foods, but the proanthocyanidins actually used for the study were isolated from cocoa. Cocoa was chosen based on recent studies which suggest that cocoa contains a level of proanthocyanidins five times higher than that found in fruits, vegetables, or tea. In addition, this research has shown that consumption of cocoa may decrease the risk of heart disease and cancer (5, 18).

Objectives

1) Determine the extent to which proanthocyanidins isolated from cocoa reduce HDC activity
2) Compare the reduction of HDC activity obtained with proanthocyanidins to the previously reported reduction in OCD activity.

Procedures and Methods

In order to determine HDC activity, histidine labeled with ^{14}C was reacted with HDC. This reaction produced histamine as well as $^{14}CO_2$ gas.

Histidine $\xrightarrow{\text{HDC}}$ Histamine + CO_2

As the $^{14}CO_2$ gas was generated, it was captured on filter paper soaked with benzethonium hydrochloride located within a small sample cup at the top of a beaker. The amount captured was measured and used to infer the amount of HDC activity within the system.

For this experiment, the system consisted of a 25 ml Erlenmeyer flask topped with a rubber stopper. A small plastic sample cup was inserted into the stopper (see Figure 3). The stoppers (#882310-0000) and the plastic sample cups (#882320-0000) were both obtained from the Kontes Glass Company of Vineland, NJ.

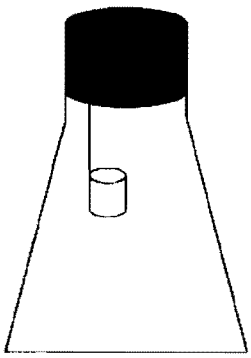

Figure 3. Experimental setup

The reaction mixtures were placed in the bottom of the flasks. A filter paper disk soaked with 20 µl of 1.0 M benzethonium hydrochloride (in ethanol) and air- dried for 10-20 minutes was placed in each sample cup. The filter papers used were S&S Filter Paper #897 Blaine test discs with a 1.27 cm diameter. The exact reaction mixture used for each experiment is outlined in Tables 1-8. Each mixture consisted of 0.1 M potassium phosphate (pH 4.5), 0.01 mM pyridoxal 5-phosphate, 1 mM L-histidine, and 0.5 µc L-histidine (Carboxyl-^{14}C) with or without enzyme and with or without inhibitor. The reaction was initiated by adding enzyme to bring the total volume to 0.5 mL. The vials were closed tightly, wrapped with parafilm to prevent loss of the stopper upon CO_2 production, and then incubated at 37°C for 90 minutes with an intermediate rate of

shaking. The incubator used in this experiment was a Nuaire IR Autoflow CO_2 Water-Jacketed incubator, and the rotator used to create shaking was a Lab-line MAX/Rotator at a speed of 2. To terminate the reaction, approximately 0.5 ml of 1.5 N $HClO_4$ was slowly added to the beakers. The beakers were further incubated with shaking for 60 minutes at 37°C. Following the final 60-minute incubation period, the paper discs were removed from the sample cups with forceps and transferred to a scintillation vial containing 10 ml of ScintiVerse scintillation solution. The samples were then measured for $^{14}CO_2$ with a Packard 1500 Tri-Carb liquid scintillation analyzer (12). Each reaction was run in triplicate, and statistical analysis was run using STATISTICA software.

The radioactive histidine used in this experiment was obtained from Perkin Elmer Life Sciences (Boston, MA), and it had a specific activity of 327 mCi/mmol. It was steri-packed at 0.1 mCi/ml in a 2:98 ethanol:water solvent. The dimer, trimer, tetramer, pentamer, and crude extract cocoa proanthocyanidins were purified and donated by the M&M Mars Corporation Hackettstown, NJ. All remaining chemicals used were obtained from the Sigma Chemical Company (St. Louis, MO).

The cocoa proanthocyanidins were extracted and isolated by the M&M Mars Corporation according to the procedure outlined by Hammerstone *et al.* In this procedure, proanthocyanidins are extracted three times with hexane to remove lipids, followed by extraction with 70% acetone in water and 70% methanol in water. The organic solvent was then evaporated with a rotary evaporator under partial vacuum at 40°C. The compounds were then isolated using high performance liquid chromatography (HPLC) in combination with mass spectrometry (MS). For the HPLC, a normal phase column with a silica stationary phase and a mobile phase containing dichloromethane, methanol, and acetic acid and water (1:1 v/v) was used. The conditions used

for included introduction of 0.05M NaCl at 0.05 ml/min just prior to entrance, a capillary voltage of 35 kV, a fragmentor voltage of 100V, a nebulizing pressure of 25 psig, and a drying temperature of 350°C (11).

Table 1. Reaction Conditions Used to Determine the Effects of Addition of Catechin

	1	2	3	4
0.1mM potassium phosphate, pH 4.5	+	+	+	+
1 mM L-histidine	+	+	+	+
0.01 mM pyridoxal-5-phosphate	+	+	+	+
0.5 μc L-histidine	+	+	+	+
20 μM catechin	-	+	-	-
200 μM catechin	-	-	+	-
0.08 units HDC	+	+	+	-

Table 2. Reaction Conditions Used to Determine the Effects of Addition of Epicatechin

	5	6	7	8	9
0.1mM potassium phosphate, pH 4.5	+	+	+	+	+
1 mM L-histidine	+	+	+	+	+
0.01 mM pyridoxal-5-phosphate	+	+	+	+	+
0.5 μc L-histidine	+	+	+	+	+
20 μM epicatechin	-	+	-	-	-
200 μM epicatechin	-	-	+	-	-
2000 μM epicatechin	-	-	-	+	-
0.08 units HDC	+	+	+	-	-

Table 3. Reaction Conditions Used to Determine the Effects of Addition of Cocoa Dimer and Trimer Proanthocyanidins.

	10	11	12	13	14	15
0.1mM potassium phosphate, pH 4.5	+	+	+	+	+	+
1 mM L-histidine	+	+	+	+	+	+
0.01 mM pyridoxal-5-phosphate	+	+	+	+	+	+
0.5 μc L-histidine	+	+	+	+	+	+
20 μM dimer	-	+	-	-	-	-
200 μM dimer	-	-	+	-	-	-
20 μM trimer	-	-	-	+	-	-
200 μM trimer	-	-	-	-	+	-
0.08 units HDC	+	+	+	+	+	-

Table 4. Reaction Conditions Used to Determine the Effects of Addition of Cocoa Tetramer, Pentamer and Crude Extract Proanthocyanidins.

	16	17	18	19	20	21	22	23
0.1mM potassium phosphate, pH 4.5	+	+	+	+	+	+	+	+
1 mM L-histidine	+	+	+	+	+	+	+	+
0.01 mM pyridoxal-5-phosphate	+	+	+	+	+	+	+	+
0.5 μc L-histidine	+	+	+	+	+	+	+	+
20 μM tetramer	-	+	-	-	-	-	-	-
200 μM tetramer	-	-	+	-	-	-	-	-
20 μM pentamer	-	-	-	+	-	-	-	-
200 μM pentamer	-	-	-	-	+	-	-	-
20 μM crude extract	-	-	-	-	-	+	-	-
200 μM crude extract	-	-	-	-	-	-	+	-
0.08 units HDC	+	+	+	+	+	+	+	-

Table 5. Reaction Conditions Used to Determine Effects of Known Inhibitor

	24	25	26
0.1mM potassium phosphate, pH 4.5	+	+	+
1 mM L-histidine	+	+	+
0.01 mM pyridoxal-5-phosphate	+	+	+
0.5 μc L-histidine	+	+	+
0.08 units HDC	-	+	+
α-fluoromehtylhistidine hydrochloride (16 mM)	-	-	+

Table 6. Reaction Conditions Used to Determine the Effects of Varying L-Histidine Concentration.

	27	28	29	30	31
0.1mM potassium phosphate, pH 4.5	+	+	+	+	+
1000 μM L-histidine	+	-	-	-	+
10 μM L-histidine	-	+	-	-	-
0. 1 μM L-histidine	-	-	+	-	-
0.01 mM pyridoxal-5-phosphate	+	+	+	+	+
0.5 μc L-histidine	+	+	+	+	+
0.08 units HDC	+	+	+	+	-

Table 7. Reaction Conditions Used to Determine the Effects of Varying Units of HDC.

	32	33	34	35
0.1mM potassium phosphate, pH 4.5	+	+	+	+
1 mM L-histidine	+	+	+	+
0.01 mM pyridoxal-5-phosphate	+	+	+	+
0.5 μc L-histidine	+	+	+	+
0.08 units HDC	+	-	-	-
0.04 units HDC[a]	-	+	-	-
0.02 units HDC[a]	-	-	+	-

[a] Triplicates of samples with 0.04 and 0.02 units of HDC were not run, but rather a single sample was run.

Table 8. Reaction Conditions Used to Determine the Effects of Using Boiled HDC.

	36	37	38
0.1mM potassium phosphate, pH 4.5	+	+	+
1 mM L-histidine	+	+	+
0.01 mM pyridoxal-5-phosphate	+	+	+
0.5 μc L-histidine	+	+	+
0.08 units HDC	+	-	-
0.08 units HDC, boiled for inactivation	-	+	-

Results and Discussion

The first HDC assay was performed at a pH of 6.9 as indicated by the method of Leinweber and Walker (1967). However, at pH 6.9, HDC activity was not detected. The lack of activity at pH 6.9 was due to the neutrality of the pH. The enzyme used in the method created by Leinweber and Walker was from a mammalian source, which had an optimum pH of 6.9. However, the enzyme used in our study was from a bacterial source, with an optimum pH of 4.5. Robertus *et al.* have reported that in a neutral pH environment, the helical structure of bacterial HDC unwinds, resulting in denaturation of the enzyme (19). For this reason, at pH 6.9 HDC was inactivated and did not show any activity. Therefore, pH was adjusted to pH 4.5 for all subsequent runs of the assay. As shown in Figure 4, this resulted in a detection of HDC activity.

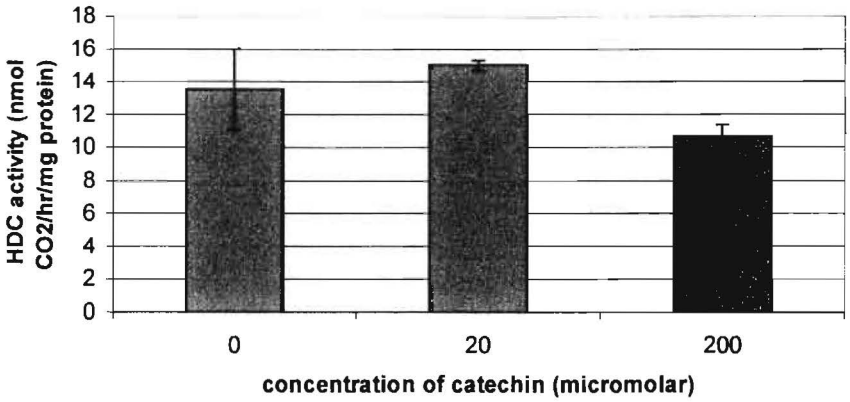

Figure 9. Effect of Catechin on HDC Activity

Once the problem with pH was resolved, the assay was run with the addition of proanthocyanidins to determine whether these compounds inhibited HDC activity. The compounds added were catechin, epicatechin, cocoa proanthocyanidins ranging from dimer to pentamer, and cocoa proanthocyanidin crude extract at concentrations of 20 μM and 200 μM (Figures 5-8). Surprisingly, the assays showed that only catechin significantly inhibited HDC activity (Figures 9-15).

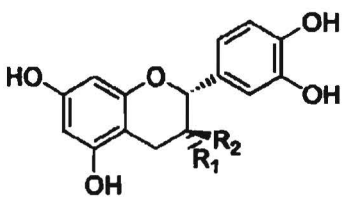

Figure 5. Catechin/Epicatechin (R1=H and R2=OH for catechin; R1=OH and R2=H for epicatechin)

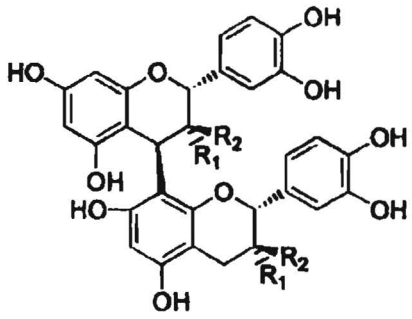

Figure 6. Cocoa (4β→8)-Dimer

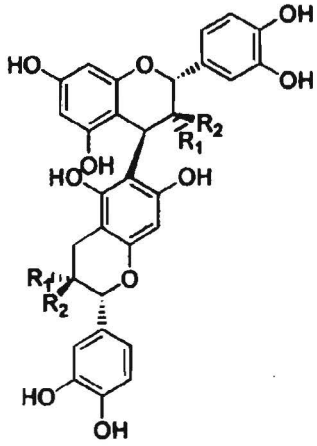

Figure 7. Cocoa (4β→6)-Dimer

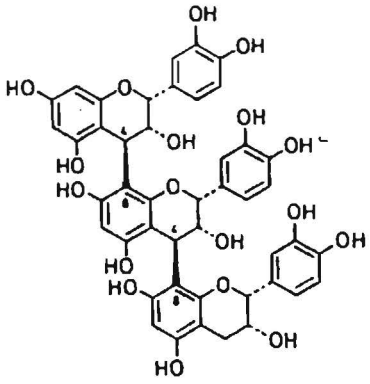

Figure 8. Cocoa Proanthocyanidin Trimer

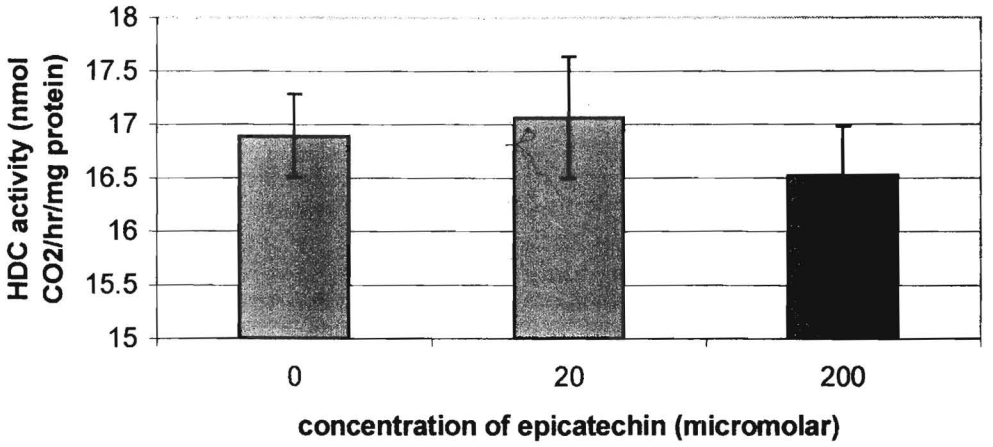

Figure 10. Effect of Epicatechin on HDC Activity

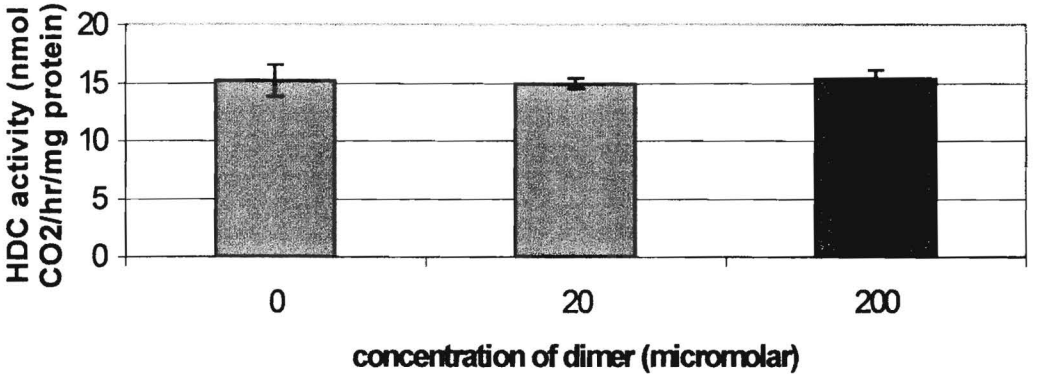

Figure 11. Effect of Cocoa Dimer on HDC Activity

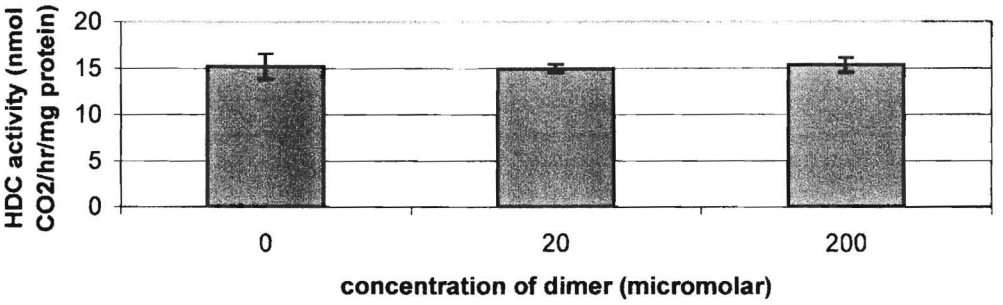

Figure 11. Effect of Cocoa Dimer on HDC Activity

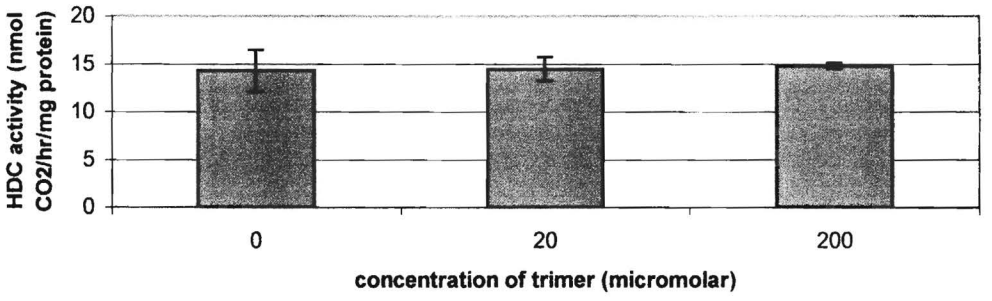

Figure 12. Effect of Cocoa Trimer on HDC Activity

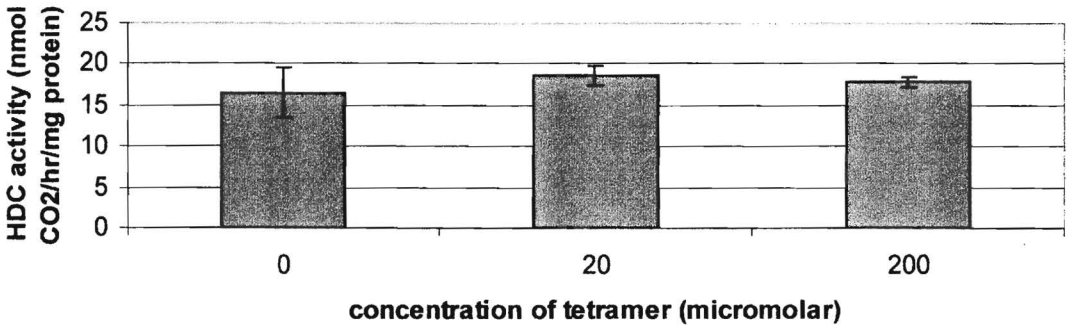

Figure 13: Effect of Cocoa Tetramer on HDC Activity

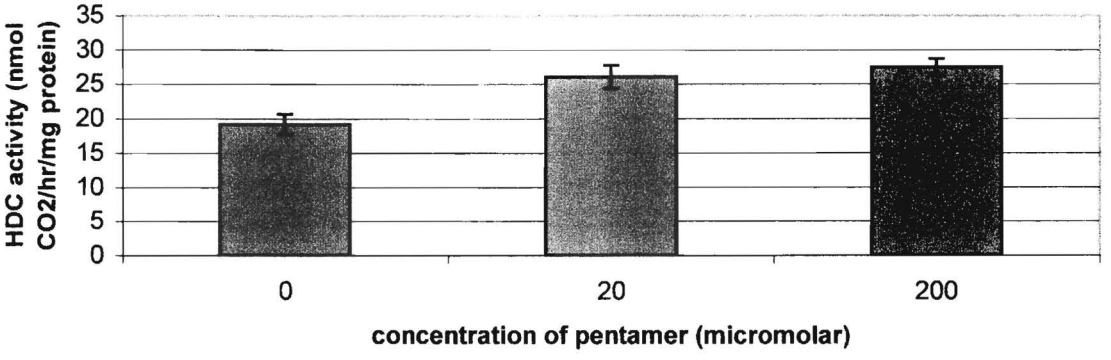

Figure 14. Effect of Cocoa Pentamer on HDC Activity

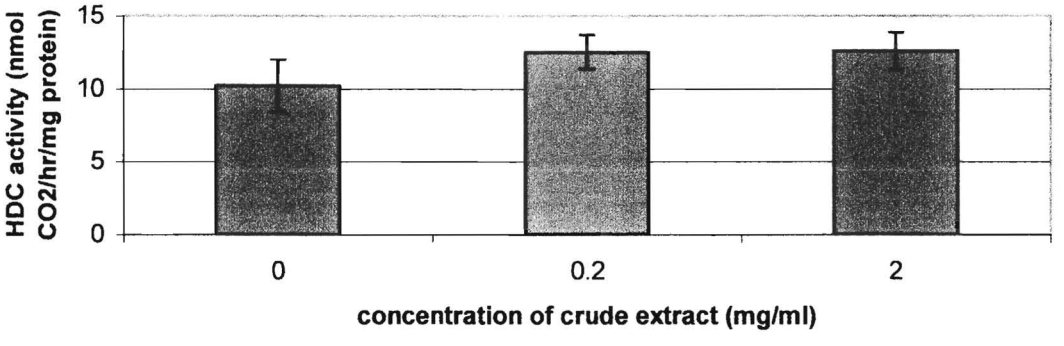

Figure 15. Effect of Cocoa Crude Extract on HDC Activity

As shown by the above graphs and the statistical data in Appendix A, HDC was significantly inhibited by only one of the proanthocyanidins tested. Addition of catechin appears to significantly lower the activity of HDC, but inhibition is only at the highest level of catechin addition, 200 μmol. In addition, though catechin appears to significantly lower HDC activity, the cocoa pentamer appears to significantly increase HDC activity at both the 20 μmol and 200 μmol levels. Because of this, it appears that proanthocyanidins cannot consistently inhibit HDC activity. In contrast, proanthocyanidins are potent inhibitors of ODC. Gali *et al* found that ODC

was significantly inhibited by catechin and epicatechin, dimer, and trimer at levels as low as10 μM. They also reported that inhibition increased with increasing polymerization (10). In this study, however, it was found that none of the compounds studied, even those with a higher degree of polymerization than those studied by Gali *et al*, were able to inhibit HDC activity at levels as high as 200 μM. In addition, Bomser *et al* reported that proanthocyanidin extracts from grape seed significantly inhibited ODC activity at levels as low as 25 μg/ml whereas the crude extract used in this experiment was unable to inhibit HDC activity at levels as high as 2 mg/ml (4).

The fact that proanthocyanidins are able to inhibit ODC but not HDC suggests that, despite the similarities between the two enzymes, some significant differences exist as well. Engel et al reported that, though they detected some important structural similarities between ODC and HDC, the protein sequence of the two enzymes did not show significant similarity. In addition, they reported that ODC appears to have a different evolutionary origin than HDC (9). For this reason, it is not hard to see how ODC and HDC may not be inhibited by the same compounds under the conditions.

This finding also supports work conducted by Zhu *et al*. In the past, researchers have suggested that proanthocyanidins non-selectively bind proteins, and it is for this reason and not because of any inherent characteristic that they are able to inhibit ODC. However, Zhu *et al* found that there is a degree of specificity to proanthocyanidin-protein interactions. They studied the ability of twenty proanthocyanidins to inhibit the binding of specific radioligands to sixteen biological receptors and found that only six of the sixteen radioligand-receptor interactions were inhibited by the proanthocyanidins (22). This experiment confirms the specificity of

proanthocyanidin-protein interactions since ODC has been effectively inhibited by proanthocyanidins while HDC was not inhibited.

After the different proanthocyanidins were added to the assay, a known inhibitor of HDC was added. This inhibitor is known as monofluromethylhistidine (MFMH) or S-(+)-α-fluoromehtylhistidine (FMH). It was added at a level of 16 mM. Again, the results were not as expected. Addition of inhibitor did not appear to significantly inhibit HDC activity (Figure 16).

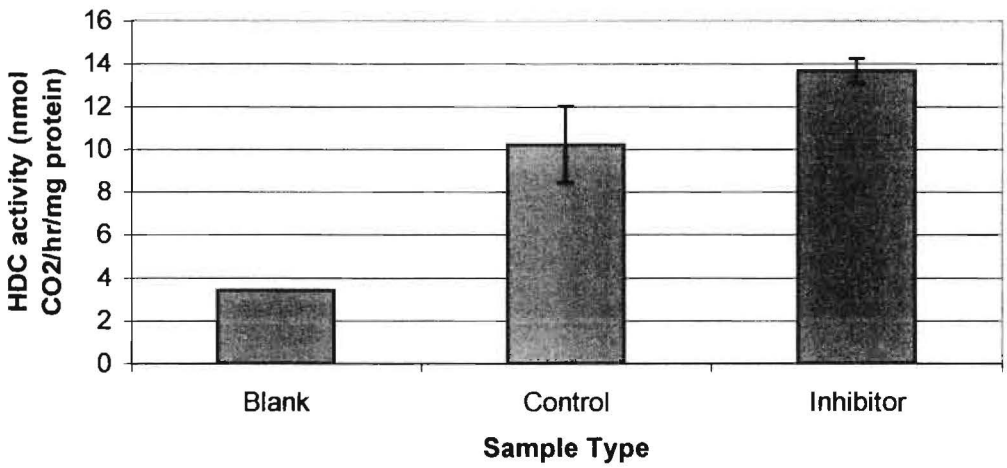

Figure 16. Effect of Inhibitor on HDC Activity

Although unexpected, this result may be explained by two different theories. First, though MFMH has been reported as a known inhibitor of HDC activity (2), this inhibition has been studied only in regard to mammalian HDC. As mentioned earlier, the HDC used in this experiment was bacterial HDC. Therefore, MFMH may be an inhibitor of only the mammalian form of HDC and not the bacterial form. Another possibility is that such an excess of enzyme was used in this experiment that the inhibitor had no effect. In other words, even though some of the enzyme may have been inhibited, there was enough remaining unaffected to carry out the

reaction as if no inhibitor were present. This is, indeed, a possibility for this specific assay due to the fact that 0.08 units of enzyme were added to each reaction, which is enough enzyme to decarboxylate 0.012 mg of protein per hour. However, only 0.008 mg of protein was added to each reaction, and the reaction was allowed to continue for one and a half hours. Certainly, the enzyme was in excess. Further characterization of the enzyme to investigate this possibility would be beneficial.

After the effects of the inhibitor were studied, the effects of varying the concentration of histidine were studied in an attempt to characterize the kinetics of the reaction. L-histidine was added at the level used for all other experiments, 1 mM (or 1000 μM), as well as at 10 μM and 0.1 μM levels. No significant difference was found in HDC activity with the different levels of HDC addition (Figure 17).

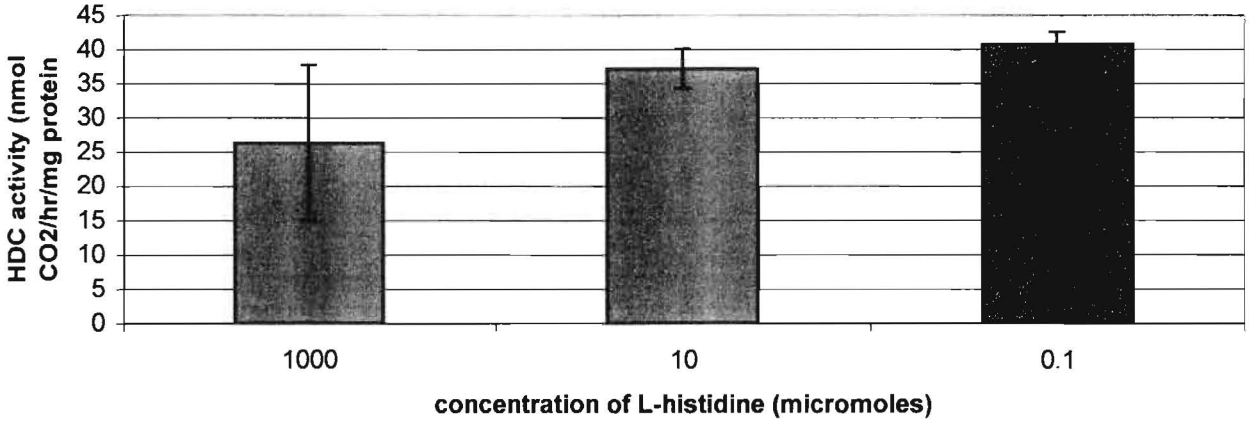

Figure 17. Effect of Varying L-Histidine Concentration on HDC Activity

This suggests that studies at levels greater than 1 mM should be conducted in order to determine the kinetics of the reaction.

The last assay conducted encompassed several different variables of interest. The first variable studied was an extension of the experiment with epicatechin. Epicatechin was added to the assay at a level of 2 mM, the highest concentration possible while still maintaining solubility of the epicatechin in water. This was meant to determine whether inhibition of HDC could be achieved with proanthocyanidins at all. The next variable studied was a variation of the blank sample. For the rest of this study, the blank sample (whose value was subtracted from all other readings) contained no enzyme or inhibitor. However, enzymologists often like to include the enzyme in an inactivated form in the blank sample to ensure all unforeseen reactions are accounted for. Therefore, a blank sample was created in which enzyme was first boiled and then added to the reaction mixture. The third variable studied was an extension of the study on varying histidine concentrations. A sample was created without adding any histidine other than the radioactively labeled compound. This was meant to help obtain more useful information about the kinetics of the reaction. Finally, samples were created in which enzyme was added at 0.04 units and 0.02 units as opposed to the 0.08 units used throughout the rest of the study. Again, this was meant to help characterize the reaction. Unfortunately, several experimental errors prevented accurate reading of these samples, and no additional conclusions can be drawn based upon the data obtained at this time.

In summary, there were two major conclusions reached in this study. First and foremost, proanthocyanidins do not appear to inhibit HDC despite the fact that they have been shown to inhibit ODC. This suggests that some important structural and/or mechanistic differences exist between the two enzymes. It also supports the work done by Zhu *et al,* which suggests that specificity does exist in proanthocyanidin-protein interactions (22). Secondly, the study shows that some important differences may exist between mammalian and bacterial HDC. This is

evidenced both by the fact that the two different forms work at different pH levels and by the fact that bacterial HDC does not appear to be inhibited by MFMH, a known inhibitor of mammalian HDC. The study also raises the possibility of further research on the topic. For instance, it would be beneficial to run further assays to characterize the kinetics of the reaction and to study whether a significant difference exists between blank samples with no enzyme and blank samples containing boiled enzyme. In addition, due to the difference in bacterial and mammalian HDC, *in vivo* studies on the inhibitory effects of proanthocyanidins on HDC should be conducted to ensure that the lack of inhibition occurs with both forms of the enzyme.

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Appendix A

Table 1. ANOVA analysis of catechin: summary of all effects.

df Effect	MS Effect	df Error	MS Error	F	p-level
2	14.50035	4	1.749589	8.287861	**.037793**

Table 2. Tukey HSD post-hoc test for catechin with the main effect being level of catechin.

	Control	20 µmol	200 µmol
Mean	13.54650	15.01033	10.68770
Control		.441894	.117588
20 µmol	.441894		**.034705**
200 µmol	.117588	**.034705**	

Table 3. ANOVA analysis of epicatechin: summary of all effects

df Effect	MS Effect	df Error	MS Error	F	p-level
2	.226806	4	.101172	2.241796	.222311

Table 4. ANOVA analysis of dimer: summary of all effects.

df Effect	MS Effect	df Error	MS Error	F	p-level
2	.101996	4	.352960	.288972	.763447

Table 5. ANOVA analysis of trimer: summary of all effects.

df Effect	MS Effect	df Error	MS Error	F	p-level
2	.211611	4	2.404127	.088020	.917468

Table 6. ANOVA analysis of tetramer: summary of all effects.

df Effect	MS Effect	df Error	MS Error	F	p-level
2	3.572226	4	3.795784	.941104	.462423

Table 7. ANOVA analysis of pentamer: summary of all effects.

df Effect	MS Effect	df Error	MS Error	F	p-level
2	58.35697	4	1.938646	30.10193	**.003881**

Table 8. Tukey HSD post-hoc test for pentamer with the main effect being level of pentamer.

	Control	20 µmol	200 µmol
Mean	19.20083	26.04897	27.43990
Control		**.008554**	**.004407**
20 µmol	**.008554**		.502224
200 µmol	**.004407**	.502224	

Table 9. ANOVA analysis of crude extract: summary of all effects.

df Effect	MS Effect	df Error	MS Error	F	p-level
2	5.463167	4	2.675103	2.042227	.244804

Table 10. ANOVA analysis of MFMH: summary of all effects

df Effect	MS Effect	df Error	MS Error	F	p-level
1	18.02363	2	2.811229	6.411300	.126945

Table 11. ANOVA analysis of varying levels of histidine: summary of all effects.

df Effect	MS Effect	df Error	MS Error	F	p-level
2	168.4288	4	46.77834	3.600572	.127525