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Inhibition of α-Amylase and Glucoamylase by Tannins Extracted from Cocoa, Pomegranates, Cranberries, and Grapes

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Supporting Information

ABSTRACT: Proanthocyanidins and ellagitannins, referred to as "tannins", exist in many plant sources. These compounds interact with proteins due to their numerous hydroxyl groups, which are suitable for hydrophobic associations. It was hypothesized that tannins could bind to the digestive enzymes α-amylase and glucoamylase, thereby inhibiting starch hydrolysis. Slowed starch digestion can theoretically increase satiety by modulating glucose "spiking" and depletion that occurs after carbohydrate-rich meals. Tannins were isolated from extracts of pomegranate, cranberry, grape, and cocoa and these isolates tested for effectiveness to inhibit the activity of α-amylase and glucoamylase in vitro. The compositions of the isolates were confirmed by NMR and LC/MS analysis, and tannin−protein interactions were investigated using relevant enzyme assays and differential scanning calorimetry (DSC). The results demonstrated inhibition of each enzyme by each tannin, but with variation in magnitude. In general, larger and more complex tannins, such as those in pomegranate and cranberry, more effectively inhibited the enzymes than did less polymerized cocoa tannins. Interaction of the tannins with the enzymes was confirmed through calorimetric measurements of changes in enzyme thermal stability.

KEYWORDS: *condensed tannins, proanthocyanidins, α-amylase inhibition, glucoamylase inhibition, pomegranate, cocoa, cranberry, grape, LC/MS, NMR, DSC*

INTRODUCTION

Modulating the blood glucose level is necessary for treating metabolic disorders such as diabetes and is also important for maintaining optimal physical and mental performance. Low blood sugar can cause fatigue, negatively affect endurance, and also impair cognitive function.[1−4] Furthermore, heavy use of easily carried and consumed, popular, high-carbohydrate foods such as energy bars and drink mixes can exacerbate the problem of insufficient blood glucose. Ingestion of high-glycemic, quickly digested carbohydrates relative to slow-digesting carbohydrates can lead to a relatively large and rapid rise in blood glucose level. Such spikes in glucose are subsequently followed by a correspondingly large release of insulin, which can then deplete glucose to levels even lower than prefeeding concentrations.[5] Since hunger and inadequate blood sugar can significantly degrade physical comfort and performance, approaches to modulate (and thus maintain) the blood glucose level would be advantageous.

One such approach could involve slowing starch digestion, in part controlled by the digestive enzymes α-amylase (AA) and glucoamylase (GA), which reduce starch polymers to oligosaccharides and oligosaccharides to glucose, respectively. Inhibition of α-amylase and/or glucoamylase would reduce the

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rate of glucose accretion into the bloodstream immediately following consumption of a starch-based meal or snack, thereby also reducing clearance of glucose by insulin. More modulated levels of glucose and insulin would theoretically help maintain satiety and comfort and thus both physical and mental performance.

Proanthocyanidins (PACs) and ellagitannins are abundant in many plant sources. Proanthocyanidins, or condensed tannins, are plant-based polymers consisting of catechin/epicatechin (flavon-3-ol) subunits. Ellagitannins are a subset of hydrolyzable tannins that comprise ellagic acid, glucose, and galloyl moieties.[6] These compounds are known to associate with some proteins through interactions with hydroxyl groups, carbonyl groups, or aromatic rings. Plant decoctions are often used in the preservation, or "tanning", of leathers because of their association with collagen.[7,8] Cranberry PACs have been shown to hinder the attachment of *Escherichia coli* to epithelium cells through interaction with proteins on the bacteria fimbriae.[9−13] Tea decoctions have been reported to inhibit salivary amylase and thus potentially reduce cavity formation due to their high levels of condensed tannins.[14,15] Furthermore, PACs/decoctions of fruit juices have been demonstrated to have potential antidiabetic benefits in rats or humans, most likely due to inhibition of digestive enzymes.[16−22] However, the tannin moiety size and structure vary widely among plant sources; furthermore, individual fruit/vegetable specimens contain a distribution of tannins/PACs. Such variation expectedly affects the likelihood and the strength of protein−tannin interaction.

Protein−tannin interactions have been investigated by employing calorimetry to study soluble tannin−protein complexes. An investigation of the interaction of hydrolyzable tannins with gelatin and bovine serum albumin (BSA) by calorimetry revealed stronger binding with gelatin than with BSA for all hydrolyzable tannins.[23,24] These studies also concluded that the magnitude of relative binding constants for protein−tannin interactions depended on the structural flexibility of the tannin molecule. A differential scanning calorimetry (DSC) study of the interaction of collagen with hydrolyzable tannins showed that the hydrothermal stability of the collagen was affected by the tannins and that the uniformity of leather tanning can be monitored by DSC.[25]

In this work, a range of tannins from different plant sources were investigated for their effectiveness to inhibite α-amylase and glucoamylase activity and to identify structural determinants of tannin−protein interaction. Tannins were isolated from pomegranate, cranberry, grape, and cocoa extracts and the compositions of these fractions confirmed by NMR and LC/MS; the results for the isolates were compared to published compositional data for the corresponding fruits/bean. The degree of enzyme inhibition by the tannins was determined by starch and maltose hydrolysis assays for α-amylase and glucoamylase, respectively. Enzyme−tannin interactions were further confirmed by characterizing by DSC the thermal stabilities of solutions containing different tannin:protein ratios.

MATERIALS AND METHODS

Safety. All work was accomplished according to standard laboratory procedures regarding use of personal protective equipment and compliance with environmental regulations.

Isolation of Condensed Tannins. Condensed tannins were extracted from whole-fruit cranberry powder (Urell, Pharmatoka), grape juice (100% Concord grape, Welch's), and cocoa powder (unsweetened Hershey's). Hydrolyzable tannins were extracted from pomegranate juice (100% pomegranate, POM Wonderful) using solid-phase chromatography according to a well-established method for tannin isolation.[26] The cranberry and cocoa powders were individually homogenized with 70% aqueous acetone and filtered, and the sediment was discarded. The collected extracts were concentrated under reduced pressure to remove acetone, suspended in water, and applied to a preconditioned C-18 solid-phase chromatography column. Juices were applied directly to the C-18 columns. The columns were washed with water to remove sugars, followed by washing with acidified aqueous methanol to remove acids. The polyphenolic fractions containing anthocyanins, flavonol glycosides, and tannins were eluted with 100% methanol and dried under reduced pressure. These fractions were suspended in 50% EtOH and applied to preconditioned Sephadex LH-20 columns that were washed with 50% EtOH to remove low molecular weight anthocyanins and flavonol glycosides. Tannins adsorbed to the LH-20 were eluted from the column with 70% aqueous acetone and monitored using diode array detection at 280 nm. The absence of absorption at 360 and 450 nm confirmed that anthocyanins and flavonol glycosides were removed. Acetone was removed under reduced pressure, and the resulting purified tannin extracts were freeze-dried. The efficacies of these methods for isolating and purifying tannin moieties from fruit extracts have been confirmed by multiple analyses, including ^{13}C NMR, electrospray mass spectrometry, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, and acid-catalyzed degradation with phloroglucinol.[9,10,26,27] The samples were maintained at −4 °C (for less than 8 weeks) until analysis.

Enzyme Activity Assays. Different assays were employed to measure the inhibitory effects of various concentrations of the tannins on α-amylase and glucoamylase activity:

***α*-Amylase Assay.** Starch hydrolysis assays incorporating the starch−iodine tests were used to monitor α-amylase activity with and without tannin. A solution of potato starch (Sigma-Aldrich, St. Louis, MO) at 15 mg/mL in pH 6.9 buffer was prepared by boiling the solution for 15 min, followed by cooling and reconstitution to the original volume. The assay medium included 5 mL of the potato starch solution and 8.6 μL of 5 mg/mL AA (α-amylase from *Bacillus lichenformis*, Sigma-Aldrich) solution, which provided approximately a 0.5% by mass enzyme-to-starch ratio. The hydrolysis reactions were performed at 55 °C. For preparation of tannin−enzyme mixtures, the enzyme concentration was kept constant at 5 mg/mL while the tannin proportion was changed to obtain tannin−AA mixtures of 1:1, 0.1:1, and 0.01:1 (w/w). For both enzyme assays, cranberry tannins were dissolved in 30% ethanol solution to ensure complete solubility. The mixtures were stirred for 1 h at ambient temperature to allow reaction between the enzyme and tannin prior to their addition to the starch medium. An approximately 0.2 mL sample was removed every 20 s from the hydrolysis reaction medium and the enzyme reaction terminated by transferring the sample into a test tube containing 1 mL of 0.5 N HCl for analysis by the starch−iodine test. Of this mixture, 0.2 mL was withdrawn and added to 5 mL of iodine solution containing 0.33 mg/mL iodine and 0.033 mg/mL KI in nanopure water. The enzyme activity was monitored using the starch−iodine method,[28] by which the absorbance of the starch−iodine solution is spectrophotometrically (model Cary 5000 spectrophotometer, Varian Co., Walnut Creek, CA) measured after 15 min against nanopure water at 620 nm. The concentration of starch in the reaction medium was calculated by comparison with a calibration curve. The initial rate of the reaction (mg of starch degraded/s/mg of protein) was determined from the slope of the initial linear portion of the starch concentration vs time curve.

Glucoamylase Assay. Glucoamylase from *Aspergillis niger*, Bradford reagent, glucose assay reagent, maltose, and bovine serum albumin were purchased from Sigma (St. Louis, MO). The protein content of the enzyme solution was determined using the Bradford reagent with BSA used as a standard. The GA concentration was maintained constant at 5 mg/mL in the GA + tannin mixtures, while the tannin proportion was changed to obtain tannin−GA mixtures of 1:1, 0.1:1, and 0.01:1 (w/w). After preparation, each mixture was

stirred for 1 h at ambient temperature for the reaction between the enzyme and tannin to take place prior to its addition to the reaction medium. For hydrolysis, 200 μL of GA or GA + tannin was added to 3.5 mL of 10 mg/mL maltose solution (in pH 5.6 acetate buffer) at 55 °C. Approximately 0.2 mL samples were removed every 40 s from the reaction medium, and the reaction was stopped by addition of 1 mL of 0.5 N HCl prior to glucose assays.

A glucose test kit (Sigma) was used to determine the glucose concentration. The sample pH was adjusted to neutral (6−7) by 0.5 N sodium hydroxide (50−200 μL) added to 1 mL of the glucose test reagent. The absorbance at 340 nm was read and the glucose concentration determined by comparison with a calibration curve. The initial rate of the reaction (mg of glucose produced/s/mg of protein) was determined from the slope of the initial linear portion of the glucose concentration vs time curve.

α-Amylase and glucoamylase assays were replicated 2−3 times.

Structural Characterization of Tannin Isolates by NMR. Approximately 20 mg of extract was added to 0.5 mL of deuterated DMSO (d_6) for analysis. The H^1 and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer (Bruker BioSpin, Billerica, MA) at 4500 and 22 000 kHz, respectively. Due to the concentration differences of PAC in the samples, the number of scans (ns) required for ^{13}C for each sample varied between 8 and 128. The 2D NMR analyses were performed to assign all proton and ^{13}C resonances using the heteronuclear multiple bond correlation with a low-pass J-filter.

Structural Characterization of Tannin Isolates by LC/Time-of-Flight (TOF)-MS and LC/MS/MS. Stock solutions of the extracts were prepared at 1500 μg/mL in methanol and diluted to 750 μg/mL with 70:30 water−methanol prior to MS analysis. An Agilent 1290 ultra-high-performance liquid chromatograph coupled to a 6224 time-of-flight mass spectrometer (Agilent Technologies, Santa Clara, CA) was used to obtain mass measurements and molecular formulas for the polyphenols and tannins present in the extracts. An Agilent 1200 liquid chromatograph coupled to a 6460 triple-quadrupole (QqQ) mass spectrometer provided structural characterization through MS/MS experiments. The same column chemistry (Zorbax Eclipse Plus C18, Agilent Technologies) and 9 min reversed-phase gradient were used for both instruments. A 5 μL volume of sample was injected on-column. The compounds were separated using mobile phases A (water, 0.1% (v/v) formic acid) and B (acetonitrile, 0.1% (v/v) formic acid) at a flow rate of 0.8 mL/min. A linear ramp increased B from 5% to 32% in 8 min. At 8.1 min, B increased to 95%, which was held for 0.9 min. The column (50 °C) was re-equilibrated at 5% B during a 3 min postrun. A 150 mm × 2.1 mm column (1.8 μm particle size) was used with the ultra-high-performance liquid chromatography (UHPLC)/TOF-MS instrument, and a 50 mm × 4.5 mm column (same particle size) was used on the LC/QqQ-MS instrument. The compounds were ionized for mass spectral detection by negative ion electrospray (ESI) at −3000 V, and TOF-MS conditions were according to Hughey et al.[29] The source conditions and ion optic settings were similar for QqQ-MS except the use of a sheath gas (11 L/min, 350 °C) to focus and desolvate the ions. A collision energy of ∼20 V and a fragmentor voltage of ∼140 V were used to fragment the ions with compounds identified by matching to standards, MS/MS spectra, comparison with published results, and/or the Phenolic Explorer database.[30] Mass Hunter software, version B.03, was used for all data acquisition and analysis.

Differential Scanning Calorimetry. Thermal analysis of α-amylase and glucoamylase incubated with each tannin:enzyme ratio of 0.01:1, 0.1:1, and 1:1 was performed by using a differential scanning calorimeter (model 2920, TA Instruments, New Castle, DE). Thermograms were recorded from 10 to 100 °C at a 5 °C/min heating rate. A 10 μL volume of 25 mg/mL α-amylase or glucoamylase and 40 μL of tannin solution (6.25, 0.625, or 0.0625 mg/mL) were mixed and incubated for 1 h prior to analysis. Cranberry specimens were evaluated in 30% ethanol solutions. Approximately 50 mg of each sample solution was loaded into the DSC pans.

RESULTS AND DISCUSSION

Hydrolysis Reaction Studies. α-amylase activity, defined as degradation of starch per second per milligram of enzyme, was calculated from starch concentration versus time data collected during hydrolysis. Figure 1A shows an example of

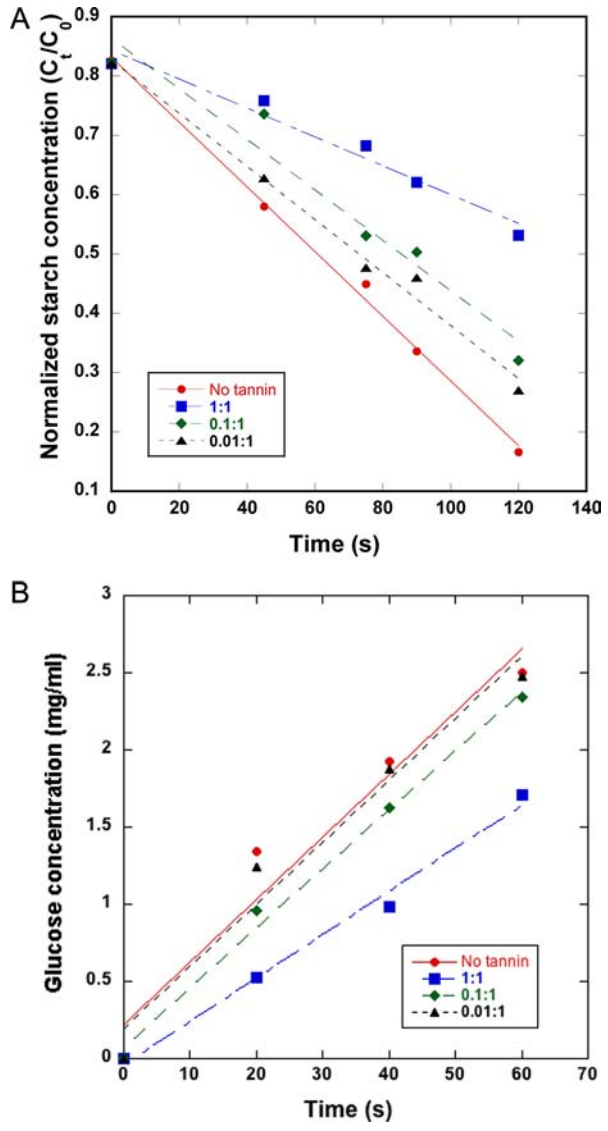

Figure 1. Representative inhibition of (A) α-amylase by different concentrations of cranberry tannins and (B) glucoamylase by different concentrations of pomegranate tannins. The figure shows increasing inhibition of the enzymes with increasing concentrations of the tannins.

starch hydrolysis data for AA with and without added tannin (from cranberries). It is apparent that the slope of the line corresponding to the rate of starch degradation decreases as the amount of tannin in the tannin−enzyme mixture increases, indicating an inhibitory effect of the tannin on AA activity. Similarly, for glucoamylase, the rates of glucose production decreased progressively with increasing tannin concentration, with the extent depending on the origin of the tannin. A representative figure, for the effect of tannin from pomegranate on glucoamylase, is shown in Figure 1B. Enzyme activity after addition of tannin was measured and calculated as a percentage of the activity of the untreated enzyme. The effects on the

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activities of the enzymes by all four tannins as a function of the tannin concentration are shown in parts A, for AA, and B, for GA, of Figure 2. In these figures a higher slope of the line

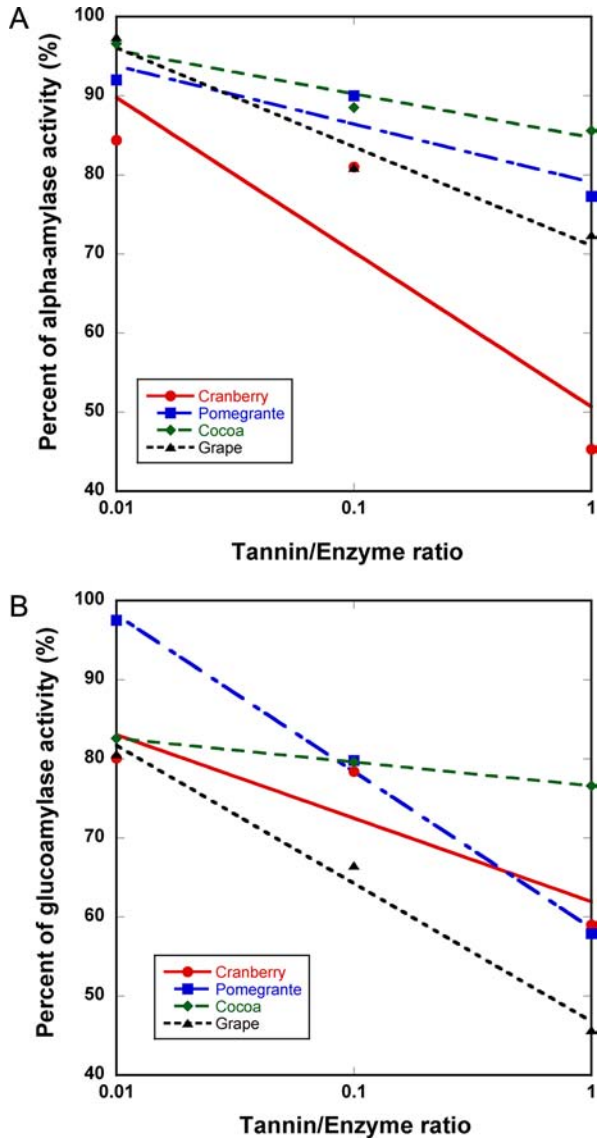

Figure 2. Inhibition of (A) α-amylase and (B) glucoamylase by 0.01:1, 0.1:1, and 1:1 tannin:enzyme ratios. The figure shows the linear dependence of activity on log(tannin concentration) but different slopes for the relationships.

indicates a greater effect of the tannin on enzyme activity. For α-amylase, cocoa, pomegranate, and grape tannins had only a slight effect at the lowest tannin:enzyme ratio (0.01) investigated. However, increasing the tannin concentrations increased inhibition of AA activity (Figure 2A). Cranberry tannins inhibited AA activity at all concentrations studied. The impact of tannins on the reduction of AA activity can be ranked from the highest to the lowest as cranberry > grape > pomegranate > cocoa.

Regarding glucoamylase, cocoa, cranberry, and grape tannins reduced GA activity approximately 20% at the 0.01:1 tannin-to-enzyme ratio. However, while increasing the proportion of cocoa tannin beyond that level did not further reduce GA activity, the three other tannins progressively inhibited the activity of this enzyme (Figure 2B).

The extents of activity inhibition by each tannin/level on each enzyme are summarized in Table 1.

NMR Studies. Due to the complexity of and the number of PAC structures in the isolates, overlapping signals with similar chemical shifts were present, creating broad peaks in the spectra. General peak identification was possible through comparison with previously published work on fruit extracts.[31,32]

H^1 NMR. The ^{1}H NMR results are shown in Figure 3A. In general, the signals from 6.4 to 7.0 ppm correspond to protons at the 2′-, 5′-, and 6′- positions, and signals from 4.8 to 5.2, from 4.2 to 4.8, and from 3.6 to 4.1 ppm correspond to protons at the 2-, 3-, and 4-carbon positions, respectively.[32] The 6-position proton signals, observed in all the isolates, were found between 5.6 and 5.9 ppm and 8-proton signals between 6 and 6.2 ppm.[31−33] Cranberry, grape, and cocoa isolates also had minimal signals contributing to the 8-proton position, suggesting PACs most likely linked with 4 → 8 bonds. Pomegranate had a stronger signal for the 8-position proton, suggesting the presence of 4 → 8 bonds as well as (majority) 4 → 6 bonds. Aromatic hydroxyl group signals from 8.5 to 9.5 ppm were apparent in all the specimens, with the signals for pomegranate shifted downfield. Several signals below 2 ppm were most likely due to lower molecular weight flavonoids.

The greatest difference among the spectra for the samples was seen for pomegranate, for which the signal at ∼3.4 ppm (typical of OH or OR and prominent in spectra for the three other specimens) was comparatively broader and less pronounced. Additionally, the sharp doublet signal near 7.5 ppm (and a small broad signal at the same position for the grape specimen) was not present in spectra for cranberry and cocoa specimens and is consistent with hydrolyzable ellagic and/or gallic acid.[33]

^{13}C NMR. The required numbers of scans for analysis were 8, 16, 128, and 128 for cocoa, pomegranate, cranberry, and grape, respectively. ^{13}C NMR spectra are shown in Figure 3B.

The pomegranate isolate was confirmed to be composed mostly of hydrolyzable tannins due to its signals at 175, 160, 140, and 113 ppm. The cranberry specimen was confirmed to be composed of mostly A-type PACs due to its signals at 151−152 and 104 ppm (in agreement with findings by Schmidt et al.[31] and Es-Safi et al.[32]). These A-linkage signals were not present in spectra for the other samples, suggesting either that the grape and cocoa specimens contained only B-type linkages or that the concentration of A-type linkages was too small to be observable. The signal between 143 and 146 ppm corresponds to either prodelphinidin (gallocatechin/epigallocatechin) or procyanidin (catechin/epicatechin) units.[31] That the pomegranate signal had a slight shift downfield suggests that this specimen contained prodelphinidin units, whereas the cranberry, cocoa, and grape specimens contained predominantly procyanidin units.

C-ring carbon signals, seen at 65−90 ppm, are typically stereochemically sensitive.[31,32] Cranberry, cocoa, and grape specimens each had a signal at 68 ppm for C3, whereas pomegranate showed a very small signal at 71 ppm. Cocoa was the only isolate to clearly show signals for C2, at 78 ppm for the *cis* form and at 81 ppm for the *trans* form. Smaller signals observed between 71 and 75 ppm for pomegranate and grape could have been due to C2 chemical shifts.

2D NMR. 2D NMR spectra (not shown) confirmed that the cranberry, cocoa, and grape specimens contained procyanidin and that the pomegranate specimen contained prodelphinidin.

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Table 1. Effect of Tannins on the Thermal Stability and Activity of AA and GA[a]

tannin	tannin:enzyme ratio	AA thermal stability increase (°C)	AA activity decrease[b] (%)	GA thermal stability increase (°C)	GA activity decrease[b] (%)
cranberry	1:1	5.0	55	1.7	41
	0.1:1	0.5	19	1.3	22
	0.01:1	−2 (decrease)	15	1.3	20
pomegranate	1:1	no change	23	1.4	42
	0.1:1	−2 (decrease)	10	1.0	20
	0.01:1	no change	8	no change	no change
grape	1:1	6.0	28	1.0	55
	0.1:1	no change	19	1.0	33
	0.01:1	no change	3	1.0	21
cocoa	1:1	2.0	14	1.0	23
	0.1:1	−5 (decrease)	10	1.0	20
	0.01:1	−5 (decrease)	1	1.0	17

[a]Results averaged over replicates. [b]Activity decrease = (1 − (activity with tannin/activity without tannin)) × 100.

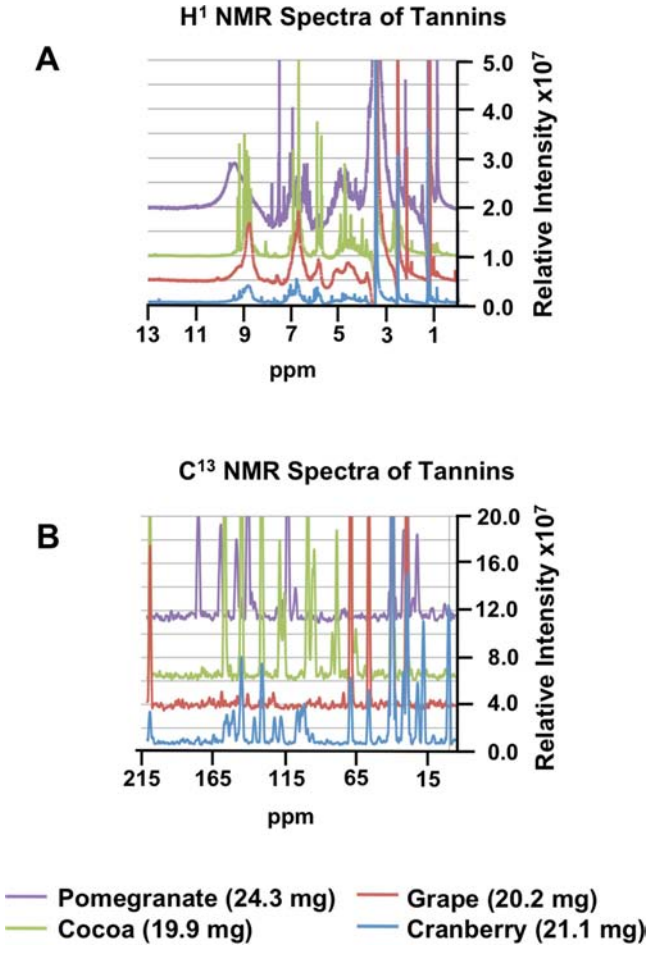

Figure 3. (A) Proton NMR spectra and (B) carbon-13 NMR spectra for the tannin extracts: pomegranate, cranberry, cocoa, and grape.

The high correlation of the pomegranate ^{13}C signals with the proton signal near 7.5 ppm indicated the presence of hydrolyzable tannins, specifically ellagitannins containing one or more ellagic acid groups. The 2D spectra furthermore confirmed that the cranberry isolate was composed primarily of A-type linkages, that the pomegranate isolate was composed mostly of 4−8 and 4−6 B-type linkages, and that the grape and cocoa isolates contained mostly 4−8 B-type linkages.

LC/MS Results. Characterization of grape, cocoa, cranberry, and pomegranate juices/extracts by LC/MS has been extensively reported, and results for our isolates are presented in light of this information for whole fruit specimens. Specifically, our goal was to distinguish the molecular weight distributions of the components present in each isolate to identify size/structure determinants that would be correlated with the ability to inhibit enzyme activity (i.e., if larger molecular weight constituents more effectively interact with active sites on the enzymes). A visual representation of the compositional differences among the specimens is shown in Figure 4, a heat map of neutral mass (Da) vs retention time (TOF-MS data). Clearly, cranberry and pomegranate, and to a lesser extent grape, show the highest diversity of constituents. Cranberry and pomegranate contained the largest tannin moieties. A total compound chromatogram (Figure 5) obtained with the UHPLC/TOF-MS provides additional compositional detail. Open shapes above the peaks signify the A-type PACs, while closed shapes signify B-type PACs. Compounds numbered 1−7 were identified by MS/MS and retention time matching to standards. As previously demonstrated in the NMR data, PACs were confirmed to be the predominate tannins observed in the isolates of grape, cocoa, and cranberry while ellagitannins were the major tannins in pomegranate.

A brief summary of structural differences is as follows.

Grape. The grape isolate contained predominately small, diverse flavonoid-type compounds with m/z < 500 (77% of the summed relative abundance across all compounds), similar to the findings of Mullen et al. for grape juice.[34] Higher molecular weight compounds were mostly B-type PACs with 2−4 (epi)catechin units. The monomers catechin and epicatechin (m/z 289) were present in approximately equal amounts as confirmed by matching to standards. One A-type procyanidin dimer (m/z 575) eluting at 4.83 min and as many as six B-type dimers (m/z 577, the most abundant eluting at 2.81 min) were present (Figure 5). The linkage type was determined according to the MS/MS fragmentation pattern.[35] Specifically, A-type PAC dimers yielded fragment ions of 449, 289, and 285, while B-type dimers yielded ions of 451, 425, 407, and 289. Trimers and tetramers were present at low relative concentrations, with B-type trimers (m/z 865) slightly more abundant than A-type trimers (m/z 863). Larger PACs and epigallocatechins, reported for grape seeds,[36,37] were not observed in the isolates.

Cocoa. The cocoa isolate consisted primarily of the monomers catechin and epicatechin, with the summed

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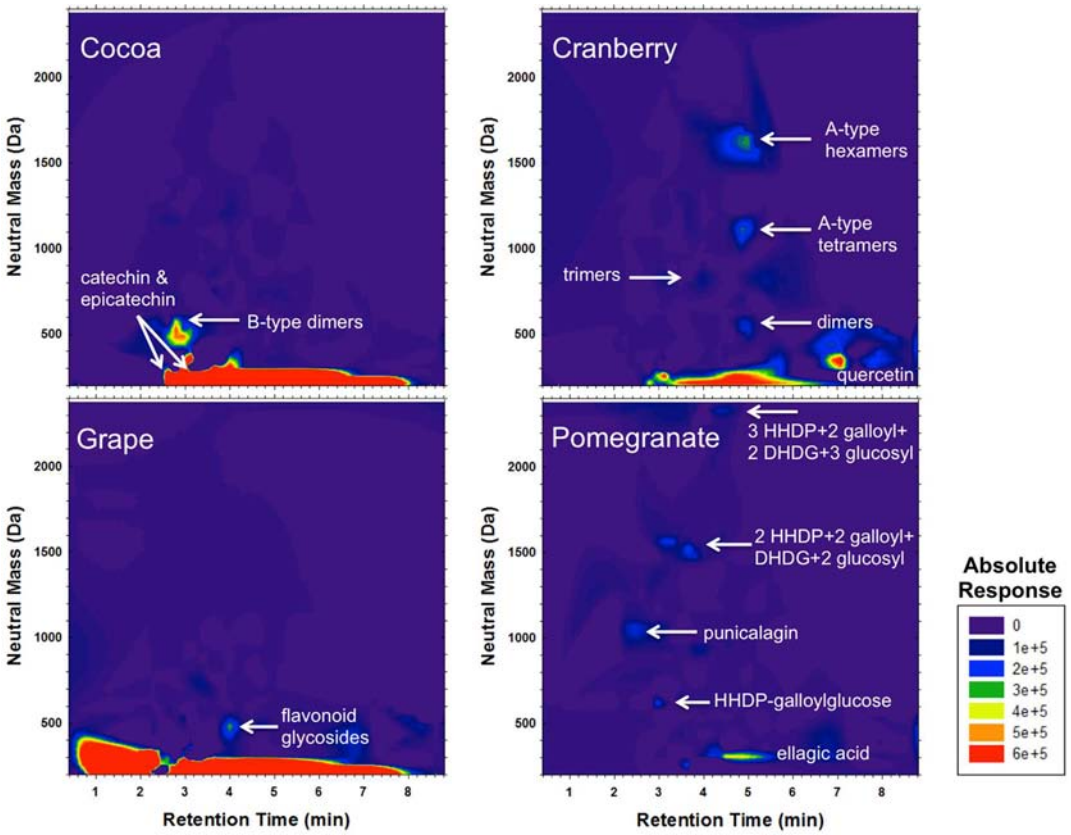

Figure 4. Heat map contour plots of extracts (negative ion ESI-TOF-MS). The graphs illustrate the diversity and presence of higher molecular weight constituents in cranberry and pomegranate extracts relative to cocoa. Red areas in the charts for cocoa and grape indicate an abundance of low molecular weight (<500) compounds over the entire retention time window.

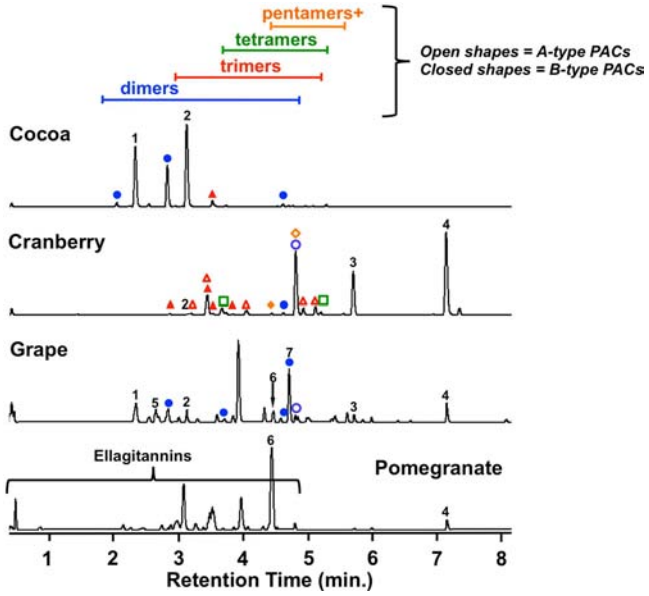

Figure 5. Total compound chromatogram (negative ion ESI-TOF-MS) for each extract with identification of PACs as dimers (blue), trimers (red), tetramers (green), and larger moieties (pentamers plus, orange). A-type and B-type PACs are marked with open and closed shapes, respectively. The pomegranate extract contained ellagitannins and no PACs. Compounds 1–7 are catechin (1), epicatechin (2), myricetin (3), quercetin (4), caffeic acid (5), ellagic acid (6), and quercetin 3-*O*-glucoside (7) as verified by matching to standards and MS/MS.

catechin/epicatechin response comprising 51% of the total response of all detected compounds. PAC B-type dimers (e.g., procyanidins B1 and B2) comprised 26% of the total response, and PAC trimers and tetramers were present in only trace amounts. Cocoa exhibited the least diversity of all extracts observed.

Cranberry. The cranberry isolate contained a high proportion of condensed tannins and relatively few lower molecular weight flavonoid compounds. Compounds with m/z > 500 comprised 46% of the total response of detected compounds; 29% of the summed response was masses >1000 Da. PACs with 2–12 polymeric units were observed with a higher proportion of A-type compounds than B-type compounds; monomers (catechin and epicatechin, m/z 289) and dimers (m/z 575, 577) were present at very low abundances. Five different A-type trimers (m/z 863) were separated and confirmed by MS/MS. A-type tetramers were present as singly and doubly charged ions, eluting at 3.67, 3.74, and 5.2 min. Larger A-type condensed tannins coeluted at 4.82 min, as evidenced by doubly charged ions at m/z 575, 863, 1151, and 1439 and 1727 that correspond to tetramers (M (neutral mass) = 1152.25 Da), hexamers (M = 1728.38 Da), octamers (M = 2304.51 Da), and decamers (M = 2880.63 Da), respectively (Supporting Information, Figure S1). Larger B-type PACs (e.g., tetramers or higher) were present in only trace amounts. The results were in accordance with citations of A-type linkages[9−12] and the presence of 10+ degrees of polymerization (DPs)[38,39] in cranberry PACs.

Pomegranate. The pomegranate extract contained primarily ellagitannins rather than condensed tannins (Figure 5). Specific

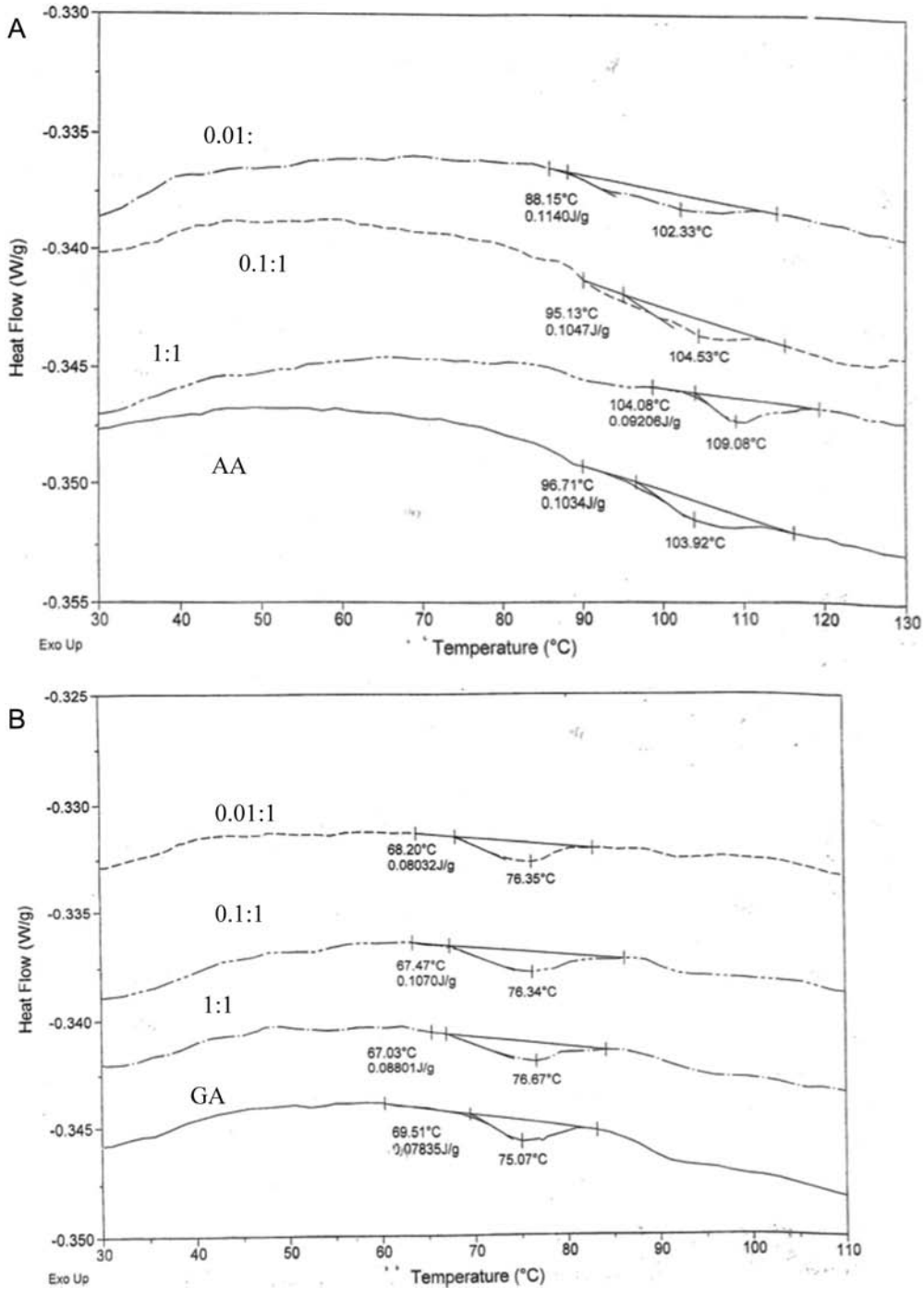

Figure 6. Representative DSC thermograms for (A) α-amylase treated with different proportions of cranberry tannin, which shows progressive broadening of denaturation endotherms with increasing tannin concentration, and (B) glucoamylase treated with different proportions of cranberry tannin, which shows a negligible effect on denaturation endotherms. Tannin-to-enzyme ratios are 1:1, 1:10, and 1:100.

identifications were made by comparison of measured masses to those previously reported for pomegranate ellagitannins,[40−44] and the results for our specimen were generally in keeping with these previous citations except for the absence of punicalgin. Like the cranberry isolate, the pomegranate specimen consisted of a larger proportion of higher molecular weight oligomers. Compounds with masses >500 Da comprised 61% of the summed response of all detected compounds; ∼29% of the summed response was masses >1000 Da. Many of the ellagitannins had two (i.e., dimer) or three (i.e., trimer) glucose units in addition to galloyl, gallagasoyl, hexahydroxydiphenoyl (HHDP), and dehydrodigalloyl (DHDG) moieties.

Calorimetry Results. Representative DSC thermograms are shown in Figure 6 for cranberry tannins incubated with α-amylase (Figure 6A) and glucoamylase (Figure 6B). In general, the effect of tannins on the thermal properties of the enzymes was dependent on the type of enzyme, the source of the tannin, and the tannin:enzyme ratio (Figure 6 and Table 1 (for all tannins)).

In most cases, increased thermal stability (evidenced by higher peak temperatures of the endotherms) due to tannin

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treatment was observed, with the greatest stabilization occurring at the highest tannin concentrations (Table 1). Increases in the endotherm temperature indicate preferential binding of tannins to native proteins, while decreasing thermal stability indicates a higher affinity of the tannin for denatured protein. No change in thermal stability indicates either a lack of binding or identical affinity of the tannins for the native and denatured forms of the enzyme.

Increased thermal stability due to binding of tannins to native protein may be associated with conformational changes effecting decreased enzyme activity. As shown in Table 1, it is evident that increases in thermal stability are observed along with decreased enzyme activity. However, in some cases, such as for grape tannin−GA mixtures, while enzyme activity decreases, the thermal stability remains relatively constant. Furthermore, for a few tannin−AA mixtures, especially those at lower tannin levels, decreased thermal stability was associated with increased enzyme activity.

In particular, DSC thermograms of AA−tannin mixtures exhibited extensive broadening of the denaturation endotherm in comparison to endotherms for the enzyme alone. Such a change in the shape of the thermal transition may indicate a change in the mechanism of denaturation, in that broadening of the endotherm suggests limited tannin-induced aggregation of the unfolded protein. Specifically, broadening of the endotherm with increased tannin content but without a change of the enthalpy of denaturation, as shown in Figure 6A, indicates that more molecular subunits of the tannin-combined enzyme, relative to those of the untreated enzyme, are involved in the denaturation process.

A similar observation was reported for collagen samples in which multiple peak DSC profiles were observed for tannin-treated collagen samples, while untreated collagen samples exhibited only a single denaturation peak.[25] A broad peak is thus interpreted as a combination of multiple, consecutive, overlapping transitions, each with a smaller heat signal.

It was also apparent from the calorimetric data that the two digestive enzymes had different mechanisms of interaction with the tannins. Similar such differences were reported for BSA and gelatin: tannins were bound to BSA nonspecifically, whereas a two-stage binding mechanism involving saturation of specific binding sites was observed for gelatin.[23] Binding of the tannins to the proteins was hypothesized to be affected by the structural flexibility of the tannin molecules.

Analytical Result Relationships. It is apparent that the tannins interacted with both enzymes. However, the results also reveal that general interaction was not entirely correlated with inhibition efficacy. It is possible that the different tannins interacted with various degrees of strength with (or associated with different sites on) the enzymes; furthermore, in the case of glucoamylase, interaction that affected the activity of the enzyme did not strongly influence its denaturation behavior.

The tannins varied in degree of inhibition efficacy in the orders cranberry > grape > pomegranate > cocoa for α-amylase and grape > pomegranate > cranberry > cocoa for glucoamylase. The inhibitory effect was strongly dependent on the tannin concentration in each case. These results are correlated with structural differences among the tannins to the extent that cocoa, the smallest and least complex of the tannins, had the least effect on the activity of either enzyme. The structural complexity or size, such as the A-type linkages and high degree of polymerization in cranberry or the association with ellagic acid in pomegranate, may have been useful conformational traits. Grape was also highly effective in inhibiting both enzymes, despite its smaller size relative to pomegranate and cranberry. This isolate, however, exhibited comparatively greater diversity, including a higher proportion of trimer units, than did cocoa. Evidently, the combination of specific molecular sizes and conformations unique to each tannin—and the corresponding molecular configuration of the active sites on each enzyme—dictated the strength and type of tannin−enzyme interactions and thus the effectiveness of inhibition.

Possible practical applications of these results stem from the finding that condensed tannins from selected plants inhibit α-amylase and glucoamylase activity in vitro, albeit to different degrees, thus slowing digestion of starch. This inhibitory effectiveness suggests the likelihood of specific dietary approaches for modulating digestion rates in vivo and thus assisting in control of blood glucose levels.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1 revealing the higher DPs for PACs as identified by negative ion ESI TOF-MS. PACs with 4+ DPs were detected as doubly charged ions as shown in zoomed mass insets. The equal mass spacings of 288 are indicative of (epi)catechin units that comprise PACs. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

AA, α-amylase; DHDG, dehydrodigalloyl; DSC, differential scanning calorimetry; GA, glucoamylase; HHDP, hexahydroxydiphenoyl; LC, liquid chromatography; NMR, nuclear magnetic resonance; QqQ MS, triple-quadrupole mass spectrometry; PAC, proanthocyanidin; TOF MS, time-of-flight mass spectrometry; TCC, total compound chromatogram

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