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Protein Binding and Astringent Taste of a Polymeric Procyanidin, 1,2,3,4,6-Penta-*O*-galloyl-β-D-glucopyranose, Castalagin, and Grandinin

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The objective of the present investigation was to examine the oral astringency and protein-binding activity of four structurally well-defined tannins, namely, procyanidin [epicatechin$_{16}$(4→8)catechin], pentagalloyl glucose (1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose), castalagin, and grandinin, representing the three main structural categories of tannins, the proanthocyanidins, the gallotannins, and the ellagitannins. Astringency threshold and dos/response were determined by the half-tongue test using a trained human panel. Protein-binding stoichiometry and relative affinity were determined using radioiodinated bovine serum albumin in precipitation or competitive binding assays. Procyanidin and pentagalloyl glucose were perceived as highly astringent compounds and had relatively steep dose/response curves, but castalagin and grandinin had a lower mass threshold for detection. In vitro, procyanidin was the most effective protein-precipitating agent and grandinin the least. Increasing the temperature increased protein precipitation by the hydrolyzable tannins, especially grandinin. All four polyphenols had higher relative affinities for proline-rich proteins than for bovine serum albumin.

KEYWORDS: Procyanidin; castalagin; grandinin; pentagalloyl glucose; PGG; astringency; protein binding; polyphenol; tannin

INTRODUCTION

The defining characteristic of the high molecular weight polyphenols known as tannins is their ability to bind and precipitate proteins (*1*). The widespread distribution of tannins in plant-based foods and beverages has motivated decades of study of their interactions with proteins. Methods used to probe the interactions between polyphenol and protein include spectroscopic, thermodynamic, and chemical techniques (*2*−*4*) for examining the soluble or insoluble complexes formed. These studies suggest that the initial binding event between tannin and protein yields soluble complexes, which upon subsequent cross-linking are transformed into insoluble precipitates (*3*, *5*). The interactions between polyphenols and proteins are the consequence of both hydrogen bonds between phenolic hydroxyl and peptide carbonyl and hydrophobic "stacking" interactions between nonpolar amino acid residues and aromatic rings of the phenolic moiety (*6*). In addition, binding is clearly affected by protein characteristics including isoelectric point, secondary/tertiary structure, and amino acid composition, with proline-rich proteins having a particularly high relative affinity for tannins (*7*).

It is widely believed that the oral sensation of astringency is a consequence of interactions between ingested tannins and salivary proline-rich proteins (*8*−*11*). The high relative affinity of proline-rich proteins for polyphenols is a consequence of the open protein structure, the exposed polypeptide backbone, and strong hydrogen-bonding properties of the tertiary amide in any amino acid−proline peptide bond (*7*, *12*). Salivary proline-rich proteins may protect mammals from the nutritional consequences of consuming tannin-rich diets (*13*). Recent studies have demonstrated that in vitro, proline-rich proteins prevent uptake of tannins by gastrointestinal cells (*14*). In vivo, salivary proline-rich proteins diminish the absorption and metabolism of dietary tannins (*15*).

Very few studies have been conducted to directly examine the role of polyphenol structure on protein binding, precipitation, or oral astringency. Broad structural features that distinguish condensed from hydrolyzable tannins cannot be used to predict ability to precipitate protein. Among both condensed and hydrolyzable tannins, the ability to precipitate protein increases as the number of catechol moieties on the polyphenol is increased (*3*, *9*). It has been suggested that structurally flexible tannins bind protein more efficiently than more rigid tannins (*16*, *17*). The polarity of the polyphenol may be important in the interaction, as suggested by the observation that the relatively hydrophobic α-anomer of 1,2,3,4,6-penta-*O*-galloyl-D-glucopy-

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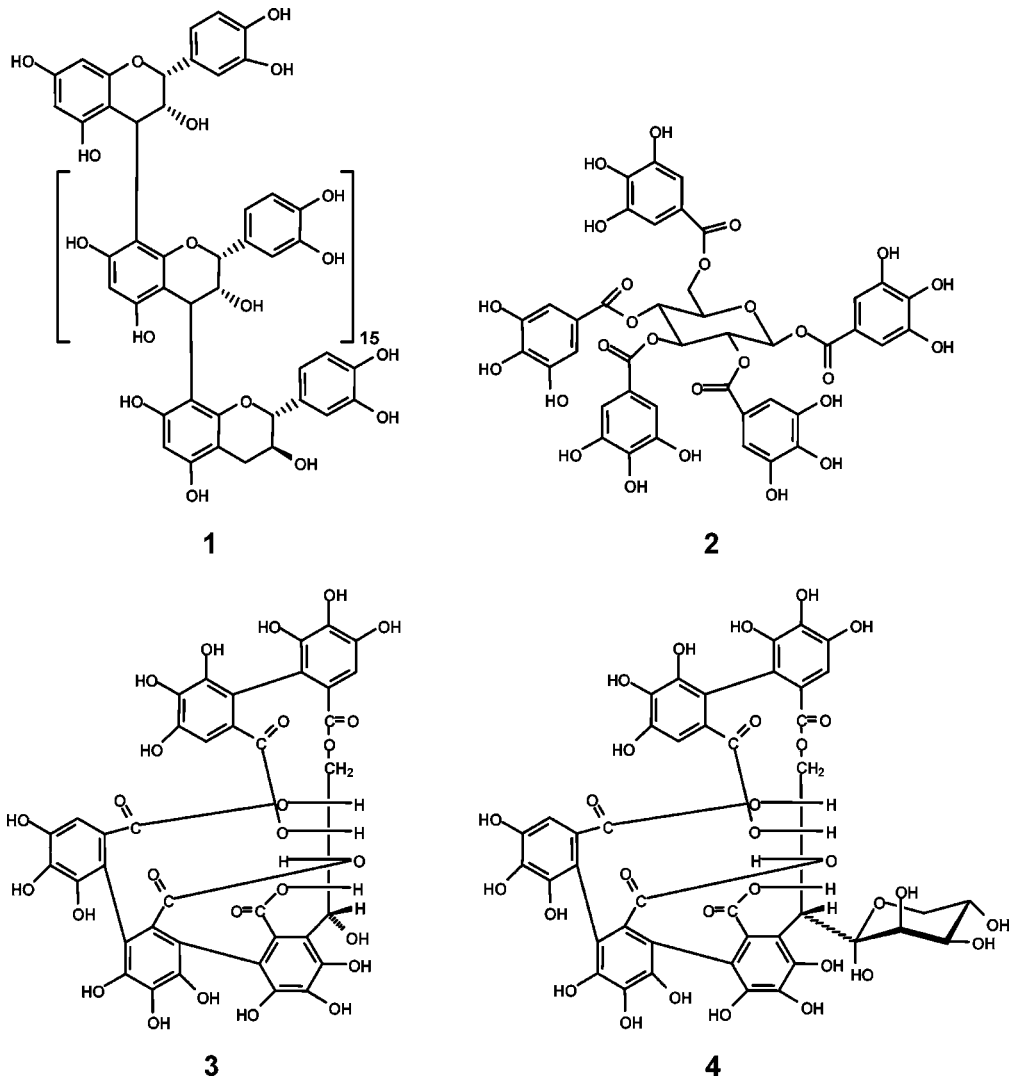

Figure 1. Chemical structures of procyanidin [epicatechin$_{16}$(4→8)catechin (**1**)], pentagalloyl glucose [1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose (**2**)], castalagin (**3**), and grandinin (**4**).

ranose has a higher affinity for BSA than the slightly more polar β-anomer (*10*). More detailed comparisons of interactions between proteins and well-defined tannins may ultimately allow reliable prediction of oral astringency and other bioactivities of tannins based on structural features.

The objective of the present investigation was to examine oral astringency and protein binding by four structurally well-defined tannins, namely, procyanidin [epicatechin$_{16}$(4→8)-catechin] (**1**) (**Figure 1**), pentagalloyl glucose (1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose) (**2**), castalagin (**3**), and grandinin (**4**). These compounds represent the three main structural categories of tannins, the proanthocyanidins, the gallotannins, and the ellagitannins. For each compound we quantitatively measured the stoichiometry of protein precipitation and relative binding affinity for a proline-rich protein. In addition, we assessed the threshold and dose/response characteristics for astringency of the four compounds using a trained human taste panel.

MATERIALS AND METHODS

Chemicals. Caffeine, gallic acid, ellagic acid, epigallocatechin-3-gallate, chloramine T, and bovine serum albumin (BSA) (fraction V, fatty acid-free) were from Sigma-Aldrich (St. Louis, MO, or Steinheim, Germany). Tannic acid and quercetin-3-*O*-β-D-galactopyranoside were obtained from Roth (Karlsruhe, Germany). Procyanidin [epicatechin$_{16}$-(4→8)catechin] (**1**) **(Figure 1**) was purified from *Sorghum* grain, and its composition and average degree of polymerization were determined by degradative cleavage (*18*). Pentagalloyl glucose (1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose) (**2**) **(Figure 1**) was purified from tannic acid, and its purity and identity were confirmed by HPLC and mass spectrometry (*5*). Wood chips produced from oak (*Quercus robur* L. and *Quercus alba* L.), which was air-dried for 2 years, were obtained from the cooperage industry (United States). Deionized water used for chromatography was purified by means of a Milli-Q Gradient A10 system (Millipore, Billerica, MA) or with a Nanopure system (Barnstead-Thermolyne, Dubuque, IA). For sensory analyses, bottled water (Evian) was adjusted to pH 4.5 with trace amounts of formic acid prior to use. All precipitation and competitive binding assays were carried out in 0.2 M acetate buffer containing 0.17 M NaCl, pH 4.9.

BSA was radioiodinated using chloramine T and Na^{125}iodine (Amersham Biosciences, Piscataway, NJ) (*19*). The labeled protein was stored at −20 °C in the acetate buffer. The protein was dialyzed (12000 MWCO) for 1−2 h at 4 °C immediately before use to ensure that all of the label was protein-bound. The specific radioactivity was adjusted to 20000 cpm per 30 μg of protein after dialysis. Calfskin gelatin (Eastman) was dissolved in the acetate buffer and diluted as needed for use in the assays.

Isolation and Purification of Castalagin and Grandinin. Oak wood chips (500 g) were extracted with 1.5 L of acetone/water (70:30 v/v) three times for 12 h with stirring. Acetone was removed, and the extract was further separated by means of adsorption chromatography and preparative RP-HPLC as described recently (*20*) to obtain the pure

ellagitannins castalagin (**3**) (**Figure 1**)and grandinin (**4**) (**Figure 1**). The purity of each ellagitannin was confirmed to be >99% by means of analytical HPLC, LC-MS, and ^{1}H NMR spectroscopy.

Protein Binding and Precipitation. Tannins were dissolved in water immediately before each experiment, and concentrations were checked spectrophotometrically on the basis of the following extinction coefficients at 280 nm: pentagalloyl glucose, 57.6 mL/mg/cm; castalagin, 22.7 mL/mg/cm; grandinin, 38.2 mL/mg/cm; procyanidin, 14.8 mL/mg/cm. The method described earlier was followed (*4*), with total reaction volumes of 400 μL for all determinations. Acetate buffer, protein, and tannin were dispensed into microfuge tubes with vortexing after each addition. The mixtures were incubated at room temperature (20 °C) or in a 40 °C water bath for 30 min and were then centrifuged at room temperature at 12000*g* for 10 min. Supernatants were removed by aspiration, and 100 μL of acetate buffer that was equilibrated at the appropriate temperature was added to each tube. Samples were not vortexed but were immediately centrifuged again for 3 min. Supernatants were aspirated, and pellets were counted in a gamma counter (Packard Instruments, Downers Grove, IL). Background binding of labeled protein to the tubes was always <10% of the total label added and was routinely subtracted during the calculations.

For stoichiometry and temperature-dependence experiments, each reaction mixture contained 30 μg of the radiolabeled BSA and 0.5−60 μg of tannin. For the competitive binding experiments, each reaction mixture contained 30 μg of the radiolabeled BSA and either 10−100 μg of unlabeled BSA or 0.2−20 μg of unlabeled gelatin. The amount of tannin used in the competitive binding experiments was different for each tannin depending on the stoichiometry of binding for that compound. For experiments with procyanidin, 0.5 μg was used; for pentagalloyl glucose, 1.5 μg was used; for castalagin, 15 μg was used; for grandinin, 30 μg was used. Each point was replicated three times, and each experiment was performed at least two independent times. Data were fit and statistically analyzed using GraphPad Prism 4.03 (GraphPad Software, Inc., San Diego, CA).

Sensory Analyses. *Panel Training.* To train the subjects to recognize and distinguish different qualities of oral sensations, 12 assessors with no history of known taste disorders (5 women and 7 men, ages 24−38 years) participated for at least 2 years in weekly training sessions. Sensory analyses were performed in a sensory panel room at 19−22 °C in three different sessions. The subjects were trained to recognize the taste of aqueous solutions (5 mL each) of the following standard compounds dissolved in bottled water (Evian; low mineralization, 500 mg/L) adjusted to pH 4.5 with aqueous formic acid (0.1%): sucrose (50 mmol/L) for sweet taste; lactic acid (20 mmol/L) for sour taste; NaCl (12 mmol/L) for salty taste; caffeine (1 mmol/L) for bitter taste; and sodium glutamate (3 mmol/L, pH 5.7) for umami taste. For puckering astringency and velvety-like astringency, the panel was trained by using gallustannic acid (0.05%) and quercetin-3-*O*-β-D-galactopyranoside (0.01 mmol/L), respectively, using the half-tongue test (*21*, *22*).

Recognition Threshold Concentrations. Threshold concentrations of astringent compounds were determined in bottled water (pH 4.5) by means of the recently developed half-tongue test (*21*, *22*) in order to overcome carry-over effects of astringent compounds. Serial 1:1 dilutions of the samples were presented in order of increasing concentrations to the trained panel of 12 persons in three different sessions, using the sip-and-spit method. At the start of the session and before each trial, the subject rinsed with water and expectorated. An aliquot (1 mL) of the aqueous solution containing the astringent compound was applied with a pipet on one side of the tongue, whereas pure water was applied on the other side of the tongue as the control. The sensory panelists were then asked to move their tongue forward and backward toward the palate for 15 s and to identify the place of astringent sensation by comparison of both sides. After indicating which part of the tongue showed the typical astringent sensation, the participant rinsed with water and, after 10 min, received another set of one blank and one taste-active sample. To prevent excessive fatigue, tasting began at a concentration level two steps below the threshold concentration that had been determined in a preliminary taste experiment. Whenever the panelist selected incorrectly, the next trial took place at the next higher concentration step. When the panelist selected correctly, the same concentration was presented again beside one blank as a confirmation of the initial response. The geometric mean of the two lowest concentrations was calculated and taken as the individual recognition threshold. The threshold value of the sensory group was approximated by averaging the threshold values of the individuals in three independent sessions. Values between individuals and separate sessions did not differ more than plus or minus one dilution step; that is, a threshold value of 1.1 μmol/L for castalagin represents a range of 0.55−2.2 μmol/L.

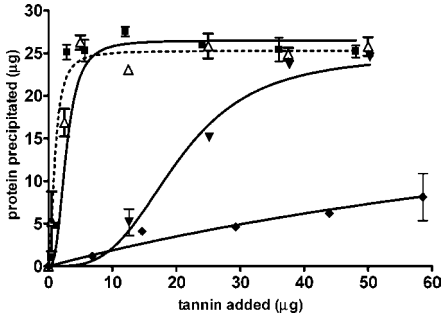

Figure 2. Protein precipitation by procyanidin (**1**) (△), pentagalloyl glucose (**2**) (■), castalagin (**3**) (▼), and grandinin (**4**) (◆) at 20 °C. Points show means of three replicates, error bars indicate standard deviation, and lines are the fits obtained by analysis of the log transformed data in GraphPad.

Recording of Human Dose/Response Functions. Serial 1:1 dilutions of the samples in water were prepared starting at the level of 256-fold above the recognition threshold concentration and ending at the concentration level two steps below the individual recognition threshold concentration. To fit the dose/response functions into a five-point intensity scale, first, the taste intensity of the individual compounds was compared at the highest concentration level by means of the half-tongue tasting method, thus offering a direct comparison of the sensory impact and a reliable evaluation of the gustatory response of different compounds. To achieve this, solutions of the individual compounds were applied in binary combinations to one side of the tongue and the assessors were asked to determine which side showed the stronger sensation (*23*). On a five-point scale with 0.25 scale subunits, a 10 mmol/L solution of epigallocatechin-3-gallate, used as the reference compound, was evaluated with the highest sensory intensity and set to the maximum score of 5.0. After the sensory intensity of each test compound at its maximum concentration had been rated, the sensory intensities of the other dilutions were determined by using the half-tongue tasting method. To achieve this, first, one dilution of an individual compound was rated against the intensity of the next lower as well as the next higher concentration of the same compound, and the intensity of this solution was approximated by comparison to the taste intensity (scores given in parentheses) of aqueous solutions containing the reference compound epigallocatechin-3-gallate in concentrations of 0.19 (0.5), 0.38 (1.0), 0.48 (1.5), 0.76 (2.0), 1.05 (2.5), 1.52 (3.0), 1.81 (3.5), 2.47 (4.0), 3.5 (4.5), and 10.0 mmol/L (5.0). Human response functions with dose-over-threshold factors on the *x*-axis and taste intensities on the *y*-axis were recorded for each individual subject in triplicates.

RESULTS AND DISCUSSION

Protein Binding and Precipitation. The four tannins examined in this study had different tendencies to precipitate protein (**Figure 2**; **Table 1**). As previously reported, protein precipitation by procyanidin was independent of temperature (**Table 1**) (*4*). There was a small but significant increase in protein precipitation by pentagalloyl glucose when the temperature was elevated to 40 °C (**Figure 3**; **Table 1**) (*4*). The hydrolyzable tannins from oak precipitate protein more effectively at elevated temperatures than at room temperature (**Table 1**). The effect of temperature was somewhat larger for castalagin and was substantial for grandinin (**Table 1**). Although as little as 1 μg of procyanidin or pentagalloyl glucose

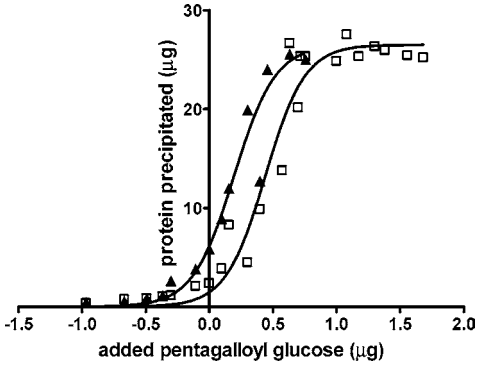

Figure 3. Precipitation of protein by pentagalloyl glucose at two temperatures. The amount of protein precipitated by various amounts of tannin was measured at 20 °C (□) and at 40 °C (▲). Points shown are the average of three determinations, and the lines are the fits to the log-transformed data.

Table 1. Precipitation of BSA by Four Tannins at Two Temperatures[a]

	PPT_{50} (μg of tannin)		apparent Hill slope		R^2 for fit	
compound	20 °C	40 °C	20 °C	40 °C	20 °C	40 °C
procyanidin (**1**)	0.98	0.93	1.6	1.8	0.81	0.96
	0.52–1.8	0.77–1.1	0.52–1.8	0.47–2.9		
pentagalloyl	2.6 a	1.6 b	2.6* x	2.4* x	0.91	0.87
glucose (**2**)	2.4–2.9	1.5–1.8	2.1–3.0	1.8–3.0		
castalagin (**3**)	20 c	12 d	3.3* y	2.4* y	0.97	0.99
	18–22	11–13	2.6–4.1	2.0–2.8		
grandinin (**4**)	220 e	25 f	0.69 z	1.0 z	0.74	0.95
	74–670	22–29	0.27–1.12	0.73–1.3		

[a] Different lower case letters indicate a statistically significant difference between temperatures for each compound ($p < 0.001$). An asterisk indicates the Hill slope is significantly different from 1.0 ($p < 0.01$). The range of values indicates the 95% confidence limit for the PPT_{50} or the apparent Hill slope.

precipitated >10% of the available protein, at least 5 μg of the oak hydrolyzable tannins was required to precipitate detectable levels of protein under the conditions of this assay.

Binding curves can be quantitatively compared by two coefficients, the EC_{50} and the Hill slope (*24*). Because protein precipitation is more complex than simple binding, we defined PPT_{50}, a parameter analogous to EC_{50}, to describe the amount of tannin required to precipitate half of the protein that is present in the assay. Precipitation data for the four tannins were analyzed using GraphPad to obtain fits with acceptable correlation coefficients (**Figure 3**; **Table 1**). For procyanidin, precipitation was independent of temperature (data not shown). All three hydrolyzable tannins precipitate protein more efficiently at 40 °C than at room temperature, indicated by a lower EC_{50} at the higher temperature (**Table 1**). For pentagalloyl glucose and castalagin, PPT_{50} was about 2-fold lower at 40 °C than at room temperature (**Table 1**). For grandinin, the difference was almost 10-fold (**Table 1**).

The Hill slope reflects the steepness of the binding curve. Ligands that bind to identical, independent sites on a protein yield a Hill slope equal to 1.0. A steeper binding curve (larger Hill slope) suggests that binding may be positively cooperative (*24*). By fitting our precipitation data we obtained values analogous to Hill slopes, and we report those as apparent Hill slopes. Castalagin and pentagalloyl glucose have apparent Hill slopes significantly larger than 1.0 (**Table 1**). Binding by procyanidin or grandinin fits a simple model with an apparent Hill slope = 1.0 (**Table 1**).

Further comparisons of binding and relative affinity were conducted at 40 °C. In addition to promoting protein binding by the hydrolyzable tannins, this temperature approaches physiological temperature. Competitive binding assays allow convenient comparison of relative binding affinity of tannins for various proteins, using a radiolabeled protein as the binding agent and other proteins as competitors. Because formation of either soluble or insoluble complexes between tannin and competitors inhibits precipitation of the tracer, this assay yields relative binding affinities (*7*). We expressed binding affinities as I_{50} values, the amount of competitor required to inhibit precipitation of the radiolabeled binding agent by 50%. It is well-established that procyanidin and pentagalloyl glucose have high relative affinity for proline-rich proteins including the salivary proline-rich proteins found in mammals (*7*, *25*, *26*). We used gelatin as a model proline-rich protein, unlabeled BSA as a control competitor, and radiolabeled BSA as the tracer.

Table 2. Competitive Binding Assays for Four Tannins[a]

		I_{50} (μg of competing protein)	
compound	tannin (μg)	BSA	gelatin
procyanidin (**1**)	0.5	29 a	3.0 x
		27–32	2.6–3.6
pentagalloyl glucose (**2**)	1.5	36 a	6.3 y
		28–46	5.2–7.5
castalagin (**3**)	15	26 a	5.5 y
		20–34	4.9–6.1
grandinin (**4**)	30	26 a	4.3 z
		24–29	3.9–4.8

[a] Different lower case letters indicate a statistically significant difference between compounds for a single protein ($p < 0.05$). For all compounds, I_{50} for gelatin was significantly less than I_{50} for BSA ($p < 0.05$). The range of values indicates the 95% confidence limit for the I_{50}.

Because each tannin has a unique binding stoichiometry (**Table 1**), a different amount of each tannin was used in the competitive binding assays (**Table 2**). As expected, when the competitor is identical to the binding agent, the I_{50} for unlabeled BSA was the same for all four tannins and was equal to the amount of radiolabeled BSA used in each assay (30 μg). This confirms that these tannins do not discriminate between radiolabeled BSA and unlabeled BSA in the binding assay and that differences in binding stoichiometry do not invalidate the method.

All four tannins have a higher relative affinity for gelatin than for BSA (**Table 2**). Procyanidin has the highest relative affinity for gelatin. The relative affinity of grandinin for gelatin is about 30% lower than that of procyanidin, whereas pentagalloyl glucose and castalagin have relative affinities that are only about half the affinity of procyanidin for gelatin (**Table 2**).

Sensory Evaluation of Ellagitannins. To evaluate the sensory quality and sensory impact of these compounds, the oral recognition threshold concentrations were determined in water (pH 4.5) using the half-mouth test for astringency (**Table 3**). The oral sensation imparted by these compounds was described as astringent and was detectable at relatively low threshold concentrations ranging from 0.2 to 1.8 μmol/L. The lowest threshold concentration for oral astringency was obtained with the monomeric ellagitannin *C*-pentoside grandinin (**Table 3**). In contrast, the monomeric ellagitannin castalagin, which lacks the pentose moiety, was detected only at a 5-fold higher threshold concentration, thus indicating that the C-glycosylation of the ellagitannin monomers enhances the astringent sensation. Pentagalloyl glucose, which is a key intermediate in ellagitannin biosynthesis (*27*), exhibited astringency at a threshold concentration very similar to the threshold found for castalagin (**Table**

Table 3. Taste Threshold Concentrations for the Astringent Sensation Induced by Four Tannins in Aqueous Solution (pH 4.5)[a]

compound	oral threshold concn	
	μmol/L	mg/L
procyanidin (**1**)	0.3	1.48
pentagalloyl glucose (**2**)	1.8	1.69
castalagin (**3**)	1.1	1.03
grandinin (**4**)	0.2	0.21

[a] Values are averages for 12 individuals, each tested in three different sessions. The values have a range of plus or minus one dilution step; that is, a threshold value of 1.1 μmol/L for castalagin represents a range of 0.55–2.2 μmol/L.

3). Procyanidin had a lower perception threshold, very similar to that of grandinin.

Human Dose/Response Functions. We recorded human dose/response functions for the four compounds to evaluate their sensory activities at different concentrations and to reveal differences in sensory behavior. Panelists often have difficulties in remembering the intensity of a taste compound for a long period of time, so the same solution of a given test compound tasted at different time intervals may be given different ratings (*28*). Consequently, recording dose/response functions with standard sensory methodologies usually leads to unreliable curves with very high error margins. To overcome this problem, we applied the recently reported half-tongue testing (*21*), offering the possibility of a direct comparison of the sensory impact of two samples. On a five-point numerical scale with 0.25 scale subunits, human dose/response functions were determined for each individual subject for pentagalloyl glucose, castalagin, grandinin, and procyanidin using standard solutions of epigallocatechin-3-gallate as the reference to define the astringent intensity represented by the individual scores (**Figure 3**). After the taste intensity of each compound at its maximum solubility had been rated, the taste intensities of the other dilutions were determined by using the half-tongue tasting method so that one dilution of an individual compound was rated against the intensity of another dilution of the same compound, and the intensity of this solution was approximated by comparison to the taste intensity of the reference compound epigallocatechin-3-gallate in defined concentrations. Human response functions with dose-over-threshold factors on the *x*-axis and taste intensities on the *y*-axis were recorded for each individual subject in triplicates. The intensity values between trained individuals and separate sessions did not differ more than ±0.4 unit on the 5-point scale (**Figure 3**).

The results, shown in **Figure 3**, clearly demonstrated that the gustatory responses for the different compounds follow rather different dose/response functions. In particular, the perception of either pentagalloyl gluocse or procyanidin yields rather steep dose/response curves and high sensory intensities at higher concentration levels. The highest intensity of 5.0 was found for an aqueous solution of pentagalloyl glucose, at a concentration 256-fold higher than its threshold concentration. Procyanidin reached an intensity of 4.0 at a concentration 128-fold higher than its threshold, with testing at higher concentrations impossible due to limited solubility. Grandinin only reached a maximum bioresponse with a score of 3.0, whereas the monomeric ellagitannin castalagin did not reach the same taste intensity as found for the three other compounds and was just perceived with an intensity score of 2.5 at a concentration 256-fold higher than the threshold concentration. Both ellagitannins exhibited a low slope for astringent intensity with ascending concentrations.

We probed the role of tannin structure in protein binding and astringency by examining four structurally defined tannins representing the three major classes of tannins found in terrestrial plants. Procyanidin (**1**) **(Figure 1**) is a simple B-1-type proanthocyanidin, with a catechin terminal unit and (4→8)-linked epicatechin units. The average degree of polymerization of the polymer isolated from sorghum grain is 17, yielding a molecular mass of about 4900 Da (*18*). The extended random coil flavan-3-ol polymers (*29*) are extremely hydrophilic as indicated by their very small octanol water partition coefficients, *P* (*30*). The log *P* for procyanidin is −2.7 (*4*). Pentagalloyl glucose (**2**) is a lower molecular mass (940 Da), more hydrophobic compound (log $P = 2.2$) (*31*) comprising five galloyl esters surrounding a core glucose. The more rigid ellagitannins form by oxidative coupling of adjacent galloyl groups in the parent compound pentagalloyl glucose. Castalagin (**3**) (934 Da) is an ellagitannin derived from pentagalloyl glucose by oxidation, glucose ring opening, and galloyl group migration (*32*). C-glycosylation of castalagin on C-1 by the pentose lyxose yields grandinin (**4**) (1066 Da) (*33*, *34*).

In our experiments, stoichiometry of binding reflects the amount of a given tannin required to precipitate a standard model protein, BSA, at pH 4.9, the optimum pH for precipitation of this protein (*35*). We have compared PPT_{50} values on a mass basis, at two temperatures, for the four structurally distinct tannins (**Table 1**; **Figure 2**). Procyanidin is a very efficient protein-precipitating agent, with the lowest PPT_{50} among the compounds we examined. On a mass basis, >1.5 times more pentagalloyl glucose than procyanidin is required to achieve PPT_{50}. On a molar basis, procyanidin is almost 10 times more effective than pentagalloyl glucose. Although pentagalloyl glucose is a more effective precipitating agent than some other simple galloyl glucoses (*36*), it is clearly a less efficient protein-binding agent than tannins such as procyanidin. The rather poor precipitating efficacies of castalagin and grandinin, namely, 7 and 14 times less effective than pentagalloyl glucose on a molar basis, may be a consequence of the rigid structures of the ellagitannins. Structural rigidity constrains cross-linking to a few specific geometries, so higher concentrations of ligand are needed to achieve aggregation. In contrast, both procyanidin and pentagalloyl glucose have flexible structures and are free to form cross-links in many different conformations.

Our data suggest that entropy-driven mechanisms such as hydrophobic interactions may have a particularly important role in the interaction of grandinin with BSA. The PPT_{50} for grandinin is 10 times lower at 40 °C than at room temperature (**Table 1**; **Figure 3**), indicating substantially stronger binding at the higher temperature. Increased binding at higher temperatures is typical for entropy-driven mechanisms such as hydrophobic interactions, but not for enthalpy-driven interactions such as hydrogen bonding. Hydrophobic interactions may play a smaller role in precipitation of BSA by pentagalloyl glucose or castalagin, because temperature has a smaller effect with these compounds. The very polar, high molecular weight procyanidin must bind to BSA almost exclusively via hydrogen bonds because protein precipitation by procyanidin is temperature independent. The extreme temperature dependence of the grandinin−protein interaction is surprising on the basis of structural considerations and measures of hydrophobicity. Grandinin is slightly more polar than castalagin (*37*) as expected, because grandinin is the *C*-glycoside of castalagin. The estimated octanol water partition coefficient for castalagin, based on its chromatographic retention factor, is intermediate between those of pentagalloyl glucose and procyanidin (*38*). Clearly, hydro-

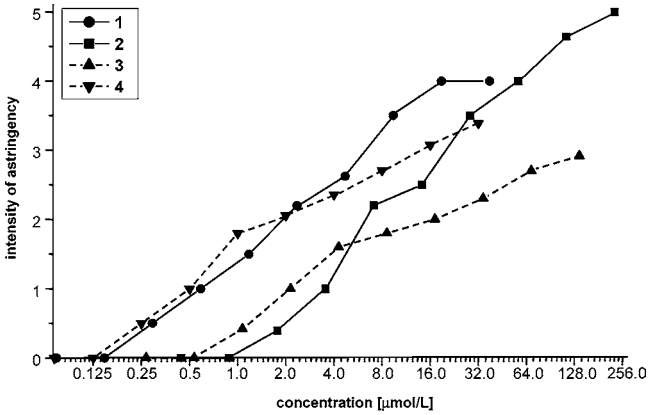

Figure 4. Human dose/response functions recorded for procyanidin (**1**), pentagalloyl glucose (**2**), castalagin (**3**), and grandinin (**4**).

phobicity is not the only characteristic that determines the tendency of a polyphenol to precipitate protein.

The temperature dependence of precipitation by gallotannins and ellagitannins is interesting in the context of foods, beverages, and biological systems, as it suggests that the tannin may not complex to protein prior to consumption when the beverage is stored at or below room temperature. When the food or beverage is consumed, its tannins may interact strongly with food, salivary, or gastrointestinal tract proteins as the ingested polyphenols reach body temperature. An earlier report that affinities become weaker as temperature is increased was not confirmed here (*3*).

Models for precipitation of protein by tannin suggest that if tannin concentration is low, soluble tannin−protein complexes form, containing 1−3 mol of tannin per mole of protein (*5*). If tannin concentration is sufficiently high, multivalent complexes form. With purified tannins, saturation stoichiometries of 20−40 mol of tannin per mole of BSA have been reported (*4*), although stoichiometries as high as 175 mol of tannin per mole of BSA have been obtained in studies using unpurified mixtures of polyphenols (*17*). We used PPT_{50} values (**Table 1**) to estimate the stoichiometry of tannin to protein in the precipitated complex, on a molar basis. The calculated stoichiometries for procyanidin, about 8 mol of procyanidin per mole of BSA, and pentagalloyl glucose, about 25 mol of pentagalloyl glucose per mole of BSA at room temperature, confirm data reported earlier (*4*). The calculated stoichiometries for castalagin (160 mol/mol) and grandinin (>5000 mol/mol) are very high, suggesting that in addition to stabilizing phenol−protein interactions, there must be numerous phenol−phenol interactions to form large colloidal aggregates (*3*).

All four polyphenols have 5−10 times higher relative affinities for proline-rich proteins than for BSA (**Table 2**). Even polyphenols with rigid structures, such as the ellagitannins examined here, bind proline-rich proteins with high relative affinity because the structural flexibility of the protein compensates for the structural rigidity of the phenolic. Affinity for proline-rich proteins is not a direct function of stoichiometry of binding. Procyanidin, the most efficient protein-precipitating tannin, and grandinin, the least efficient protein precipitant, shared high relative affinities for proline-rich protein. Pentagalloyl glucose and castalagin had somewhat lower relative affinities for the proline-rich protein.

We propose that the astringent response is a combined function of the ability of a given tannin to bind soluble proteins such as BSA and its tendency to interact with proteins via hydrophobic binding. The taste panel assigned a relatively high threshold concentration (**Table 3**) for detecting the astringency of procyanidin and pentagalloyl glucose, which are very effective protein-precipitating agents. Castalagin and grandinin, which are less effective at precipitating BSA, were detected at lower levels by the taste panel. The entropically driven component of binding, which is characteristic of castalagin and grandinin, suggests that these tannins may preferentially bind to hydrophobic constituents of the mouth rather than to soluble salivary proteins and that binding to these components may elicit the astringent response. Tannins such as pentagalloyl glucose and procyanidin may selectively bind soluble proteins and only associate with membranes when present at high concentrations, resulting in a relatively large taste threshold. The steep taste dose/response for procyanidin and pentagalloyl glucose (**Figure 4**) may reflect the ability of these tannins to saturate soluble proteins and then bind membrane-bound proteins very efficiently. Castalagin and grandinin may have flat dose/response curves because they bind and saturate the membrane required for the astringent sensation at relatively low concentration. Further structure−activity studies are needed to validate and understand the relationship between the chemical structure of a polyphenol, its protein-binding activity, and its oral astringency impact.

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LITERATURE CITED

(1) Bate-Smith, E. C.; Swain, T. In *Flavonoid Compounds; Comparative Biochemistry*; Mason, H. S., Florkin, A. M., Eds.; Academic Press: New York, 1962; pp 755−809.

(2) Baxter, N. J.; Lilley, T. H.; Haslam, E.; Williamson, M. P. Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. *Biochemistry* **1997**, *36*, 5566−5577.

(3) Charlton, A. J.; Baxter, N. J.; Khan, M. L.; Moir, A. J. G.; Haslam, E.; Davies, A. P.; Williamson, M. P. Polyphenol/peptide binding and precipitation. *J. Agric. Food Chem.* **2002**, *50*, 1593−1601.

(4) Hagerman, A. E.; Rice, M. E.; Ritchard, N. T. Mechanisms of protein precipitation for two tannins, pentagalloyl glucose and epicatechin16-(4→8)-catechin (procyanidin). *J. Agric. Food Chem.* **1998**, *46*, 2590−2595.

(5) Chen, Y.; Hagerman, A. E. Characterizing soluble non-covalent complexes between bovine serum albumin and β-1,2,3,4,6-penta-*O*-galloyl-D-glucopyranose by MALDI-TOF mass spectrometry. *J. Agric. Food Chem.* **2004**, *52*, 4008−4011.

(6) Haslam, E. *Practical Polyphenolics: from Structure to Molecular Recognition and Physiological Function*; Cambridge University Press: Cambridge, U.K., 1998.

(7) Hagerman, A. E.; Butler, L. G. Specificity of proanthocyanidin−protein interactions. *J. Biol. Chem.* **1981**, *256*, 4494−4497.

(8) Jobstl, E.; O'Connell, J.; Fairclough, J. P. A.; Williamson, M. P. Molecular model for astringency produced by polyphenol/protein interactions. *Biomacromolecules* **2004**, *5*, 942−949.

(9) Luck, G.; Liao, H.; Murray, N. J.; Grimmer, H. R.; Warminski, E. E.; Williamson, M. P.; Lilley, T. H.; Haslam, E. Polyphenols, astringency and proline-rich proteins. *Phytochemistry* **1994**, *37*, 357−371.

(10) Feldman, K. S.; Sambandam, A.; Lemon, S. T.; Nicewonger, R. B.; Long, G. S.; Battaglia, D. F.; Ensel, S. M.; Laci, M. A. Binding affinities of gallotannin analogs with bovine serum albumin: ramifications for polyphenol-protein molecular recognition. *Phytochemistry* **1999**, *51*, 867−872.

(11) Edelmann, A.; Lendl, B. Toward the optical tongue: flow-through sensing of tannin-protein interactions based on FTIR spectroscopy. *J. Am. Chem. Soc.* **2002**, *124*, 14741−14747.

(12) Simon, C.; Barathieu, K.; Laguerre, M.; Schmitter, J. M.; Fouquet, E.; Pianet, I.; Dufourc, E. J. Three-dimensional structure and dynamics of wine tannin−saliva protein complexes. A multitechnique approach. *Biochemistry* **2003**, *42*, 10385−10395.
(13) Mehansho, H.; Butler, L. G.; Carlson, D. M. Dietary tannins and salivary proline-rich proteins: Interactions, induction and defense mechanisms. *Annu. Rev. Nutr.* **1987**, *7*, 423−440.
(14) Cai, K.; Hagerman, A. E.; Minto, R. E.; Bennick, A. Decreased polyphenol transport across cultured intestinal cells by a salivary proline-rich protein. *Biochem. Pharmacol.* **2006**, *71*, 1570−1580.
(15) Skopec, M. M.; Hagerman, A. E.; Karasov, W. H. Do salivary proline-rich proteins counteract dietary hydrolyzable tannin in laboratory rats? *J. Chem. Ecol.* **2004**, *30*, 1679−1692.
(16) Haslam, E. In *Polyphenol Complexation; Polyphenolic Phenomena*; Scalbert, A., Ed.; INRA: Paris, France, 1993; pp 23−32.
(17) Frazier, R. A.; Papadopoulou, A.; Mueller-Harvey, I.; Kissoon, D.; Green, R. J. Probing protein−tannin interactions by isothermal titration microcalorimetry. *J. Agric. Food Chem.* **2003**, *51*, 5189−5195.
(18) Schofield, J. A.; Hagerman, A. E.; Harold, A. Loss of tannins and other phenolics from willow leaf litter. *J. Chem. Ecol.* **1998**, *24*, 1409−1421.
(19) Greenwood, F. C.; Hunter, W. M.; Glover, J. S. The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. *Biochem. J.* **1963**, *89*, 114−123.
(20) Glabasnia, A.; Hofmann, T. Sensory-directed identification of taste-active ellagitannins in American (*Quercus alba* L.) and European oak wood (*Quercus robur* L.) and quantitative analysis in bourbon whiskey and oak-matured red wines. *J. Agric. Food Chem* **2006**, *54*, 3380−3390.
(21) Scharbert, S.; Holzmann, N.; Hofmann, T. Identification of the astringent taste compounds in black tea by combining instrumental analysis and human bioresponse. *J. Agric. Food Chem.* **2004**, *52*, 3498−3508.
(22) Scharbert, S.; Jezussek, M.; Hofmann, T. Evaluation of the taste contribution of theaflavins in black tea infusions using the taste activity concept. *Eur. Food Res. Technol.* **2004**, *218*, 442−447.
(23) Stark, T.; Hofmann, T. Structures, sensory activity, and dose/response functions of 2,5-diketopiperazines in roasted cocoa nibs (*Theobroma cacao*). *J. Agric. Food Chem.* **2005**, *53*, 5419−5428.
(24) Motulsky, H.; Christopoulos, A. *Fitting Models to Biological Data Using Linear and Nonlinear Regression: A Practical Guide to Curve Fitting*; Oxford University Press: New York, 2004; pp 351.
(25) Murray, N. J.; Williamson, M. P.; Lilley, T. H.; Haslam, E. Study of the interaction between salivary proline-rich proteins and a polyphenol by ^{1}H-NMR spectroscopy. *Eur. J. Biochem.* **1994**, *219*, 923−935.
(26) Mehansho, H.; Hagerman, A.; Clements, S.; Butler, L.; Rogler, J.; Carlson, D. M. Modulation of proline-rich protein biosynthesis in rat parotid glands by sorghums with high tannin levels. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 3948−3952.
(27) Niemetz, R.; Gross, G. G. Oxidation of pentagalloylglucose to the ellagitannin, tellimagrandin II, by a phenol oxidase from *Tellima grandiflora* leaves. *Phytochemistry* **2003**, *62*, 301−306.
(28) Noble, A. C. In *Chemistry of Taste—Mechanism, Behaviours, and Mimics*; Given, P., Paredes, D., Eds.; American Chemical Society: Washington, DC, 2002; No. 825, pp 192−201.
(29) Bergmann, W. R.; Viswanadhan, V. N.; Mattice, W. L. Conformations of polymeric proanthocyanidins composed of (+)-catechin or (−)-epicatechin joined by 4→6 interflavan bonds. *J. Chem. Soc., Perkin Trans. 2* **1988**, 45−47.
(30) Leo, A.; Hansch, C.; Elkins, D. Partition coefficients and their uses. *Chem. Rev.* **1971**, *71*, 525−616.
(31) Tanaka, T.; Zhang, H.; Jiang, Z. H.; Kouno, I. Relationship between hydrophobicity and structure of hydrolyzable tannins, and association of tannins with crude drug constituents in aqueous solution. *Chem. Pharm. Bull.* **1997**, *45*, 1891−1897.
(32) Helm, R. F.; Zhentian, L.; Ranatunga, T.; Jervis, J.; Elder, T. Toward understanding monomeric ellagitannin biosynthesis. In *Plant Polyphenols 2. Chemistry, Biology, Pharmacology, Ecology*; Gross, G. G., Hemingway, R. W., Yoshida, T., Eds.; Kluwer Academic/Plenum Publishers: New York, 1999; Vol. 66, pp 83−99.
(33) Puech, J. L.; Mertz, C.; Michon, V.; Le Guerneve, C.; Doco, T.; Du Penhoat, C. H. Evolution of castalagin and vescalagin in ethanol solutions. Identification of new derivatives. *J. Agric. Food Chem.* **1999**, *47*, 2060−2066.
(34) Viriot, C.; Scalbert, A.; du Penhoat, C. L. M. H.; Moutounet, M. Ellagitannins in woods of sessile oak and sweet chestnut dimerization and hydrolysis during wood ageing. *Phytochemistry* **1994**, *36*, 1253−1260.
(35) Hagerman, A. E.; Butler, L. G. Protein precipitation method for the determination of tannins. *J. Agric. Food Chem.* **1978**, *26*, 809−812.
(36) McManus, J. P.; Davis, K. G.; Beart, J. E.; Gaffney, S. H.; Lilley, T. H.; Haslam, E. Polyphenol interactions. Part 1. Introduction; some observations on the reversible complexation of polyphenols with proteins and polysaccharides. *J. Chem. Soc., Perkin Trans. 2* **1985**, 1429−1438.
(37) DuPenhoat, C. L. M. H.; Michon, V. M. F.; Peng, S. Y.; Viriot, C.; Scalbert, A.; Gage, D. Structural elucidation of new dimeric ellagitannins from *Quercus robur* L.—roburin-a, roburin-B, roburin-C, roburin-D, and roburin-E. *J. Chem. Soc., Perkin Trans. 1* **1991**, 1653−1660.
(38) Tang, H. R.; Covington, A. D.; Hancock, R. A. Structure−activity relationships in the hydrophobic interactions of polyphenols with cellulose and collagen. *Biopolymers* **2003**, *70*, 403−413.

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