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Hydrolyzable Tannin Structures Influence Relative Globular and Random Coil Protein Binding Strengths

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Binding parameters for the interactions of pentagalloyl glucose (PGG) and four hydrolyzable tannins (representing gallotannins and ellagitannins) with gelatin and bovine serum albumin (BSA) have been determined from isothermal titration calorimetry data. Equilibrium binding constants determined for the interaction of PGG and isolated mixtures of tara gallotannins and of sumac gallotannins with gelatin and BSA were of the same order of magnitude for each tannin (in the range of 10^4-10^5 M^{-1} for stronger binding sites when using a binding model consisting of two sets of multiple binding sites). In contrast, isolated mixtures of chestnut ellagitannins and of myrabolan ellagitannins exhibited 3−4 orders of magnitude greater equilibrium binding constants for the interaction with gelatin ($\sim 2 \times 10^6$ M^{-1}) than for that with BSA ($\sim 8 \times 10^2$ M^{-1}). Binding stoichiometries revealed that the stronger binding sites on gelatin outnumbered those on BSA by a ratio of at least ~2:1 for all of the hydrolyzable tannins studied. Overall, the data revealed that relative binding constants for the interactions with gelatin and BSA are dependent on the structural flexibility of the tannin molecule.

KEYWORDS: Hydrolyzable tannin; polyphenol; protein; albumin; gelatin; isothermal titration calorimetry

INTRODUCTION

Tannins are usually defined as polyphenolic compounds that precipitate proteins and are part of a diverse group of polyphenols that are formed as secondary metabolites in plants (*1*, *2*). Tannins comprise a wide range of oligomeric and polymeric polyphenols; condensed tannins (syn. proanthocyanidins), gallotannins, and ellagitannins are the most widely occurring tannins. The gallotannins and ellagitannins are also known as hydrolyzable tannins.

Tannins exhibit numerous biological activities that are of interest in human and veterinary medicine (*2*, *3*). Many herbal medicines contain gallo- and ellagitannins, which are thought to be active ingredients (*4*), and have been used since antiquity to improve vascular health and for the treatment of cancer, respiratory, and many other diseases (*2*, *5*−*8*). It is probable that the interaction of tannins with proteins is fundamental to their observed biological activities (*3*, *9*, *10*); yet, despite many years of study, there are still many unanswered questions and unresolved contradictions in the literature. Therefore, a better understanding of this interaction will enable clearer rationalization of the biological activities of tannins.

It has long been known that relative affinities of tannins for different proteins can vary as much as 10000-fold (*11*, *12*) and that tannins bind preferentially to proline-rich proteins that have either random coil or collagen-like helical conformations (*11*, *13*). Tannins are thought to act as multidentate ligands to facilitate protein cross-linking and consequent precipitation (*14*, *15*), which has led to the inference that high molecular weight tannin molecules should precipitate proteins more effectively (*6*), although some data suggest that this rule is an oversimplification and does not apply to all tannins (*16*). For example, in an enzyme-linked immunosorbent assay (ELISA)-based competition assay, it was found that higher molecular weight pentagalloyl glucose (PGG) and rugosin D ellagitannins interacted less strongly with proline-rich proteins than the lower molecular weight tetragalloyl glucose (*17*).

Tannin−protein interactions have been studied by using competitive binding assays in which an unlabeled test protein competes with a standard labeled protein to inhibit its binding and precipitation with tannin (*11*, *18*) or by characterizing precipitated tannin−protein complexes (*19*). However, precipitation may not occur in biological systems where the stoichiometries of the interacting species would tend to lead to soluble complexes only. Therefore, methods to study soluble tannin−protein complexes have become the focus of more recent studies. Such methods have included equilibrium dialysis (*14*, *15*), size exclusion chromatography (*20*), nuclear magnetic resonance

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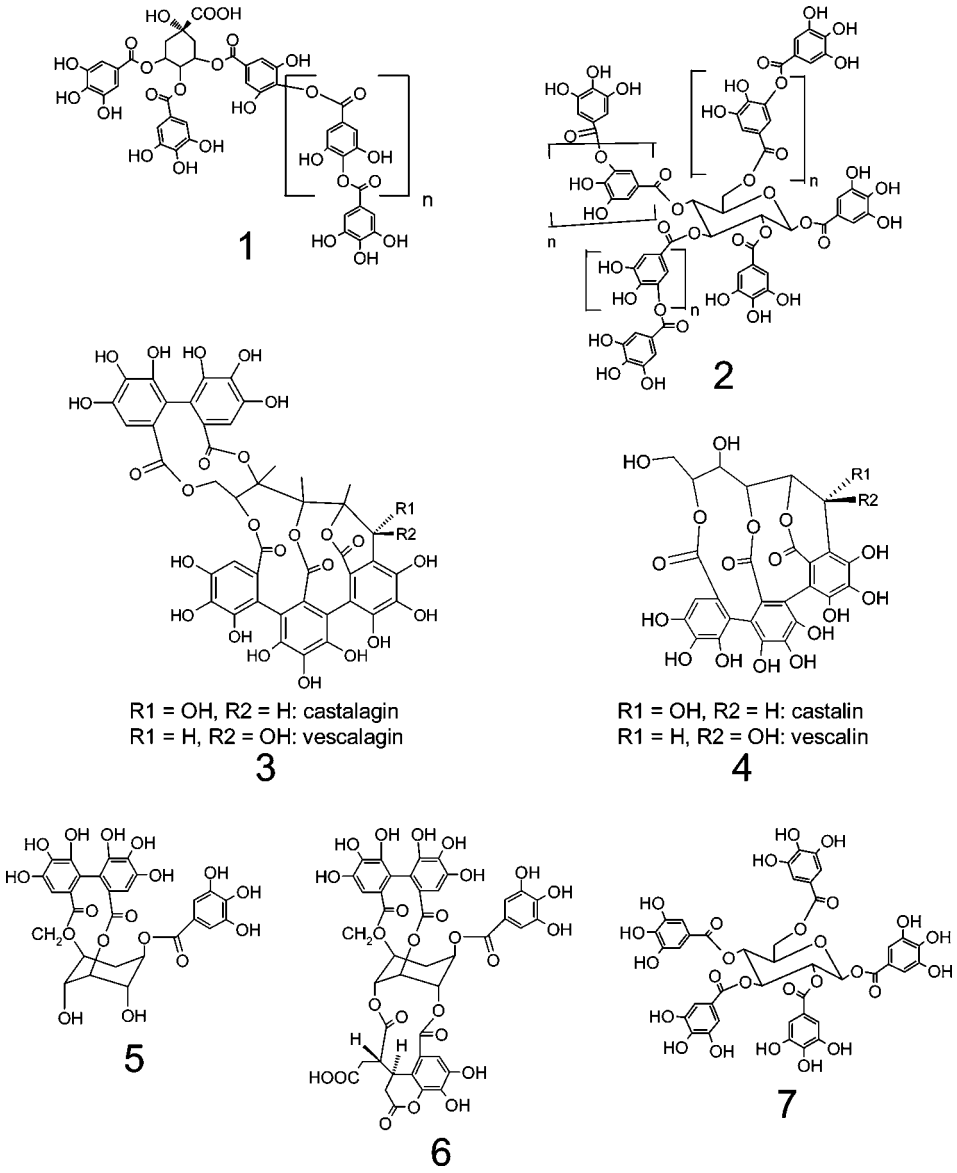

Figure 1. Chemical structures of tara gallotannins (**1**), sumac gallotannins (**2**), chestnut ellagitannins (**3** and **4**), myrabolan ellagitannins [corilagin (**5**) and chebulagic acid (**6**)], and PGG (**7**). Structures **1**–**6** are well-described in the literature and were confirmed by MALDI-TOF mass spectrometry of the isolated tannin mixtures.

(NMR) spectroscopy (*21*−*24*), electrospray ionization mass spectrometry (*25*, *26*), matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (*27*, *28*), and isothermal titration calorimetry (ITC) (*27*, *29*−*31*). Of these methods, ITC is unique in that it enables the direct measurement of the thermodynamics of protein−ligand interactions (*32*, *33*). All reversible biomolecular interactions involve changes in enthalpy (i.e., the liberation or absorption of heat energy); thus, ITC can be universally applied to their study (*33*). Indeed, the technique is particularly attractive since it can be used to measure the strength and stoichiometry of an interaction in solution and without chemical modification or immobilization of either interacting species. It is also tolerant to any precipitation that may occur during the interaction and does not pose limitations in terms of the molecular size of interacting species.

In the present study, ITC has been employed to characterize the binding of PGG and four hydrolyzable tannins (**Figure 1**), representing gallotannins and simple ellagitannins, to gelatin and bovine serum albumin (BSA). Gallotannins are considered to be structurally more flexible in comparison to the more rigid ellagitannins, which contain intramolecular biphenyl linkages (*1*, *2*). Gelatin is proline-rich, has an open random coil conformation, and is a model for seed prolamins and salivary proline-rich proteins, the latter being the focus of recent research into the molecular basis for astringency (*34*). BSA is a well-characterized globular protein. Both gelatin and BSA have been commonly used in the literature for investigation of relative binding affinities of tannins.

MATERIALS AND METHODS

Materials. BSA (purity ≥99%, essentially globulin free, 66 kDa) and bovine skin gelatin (100 kDa) were purchased from Sigma (Poole, Dorset, United Kingdom). PGG was used as provided and was isolated as a pure compound from commercially available tannic acid (Coleman & Bell Co., Norwood, OH) via methanolysis as described previously with an overall mass yield of 340 mg PGG/g tannic acid (*28*). The PGG structure was confirmed by mass spectrometry and ^{1}H NMR spectroscopy, and the material was homogeneous by high-performance liquid chromatography (HPLC). Tara, sumac, chestnut, and myrabolan tannins were isolated from commercially available tannin products donated by Forestal Quebracho Ltd. (Reading, Berkshire, United Kingdom) and were mixtures of closely related tannins with a range

of molecular weights. All solutions for ITC analysis were prepared in 50 mM citrate buffer at pH 6 and were degassed under vacuum prior to use.

Isolation of Tannins. Tannins were isolated from commercially available tannin products by chromatography on Sephadex LH-20 (Amersham Biosciences, Chalfont St. Giles, Buckinghamshire, United Kingdom). Commercial tannin products (2 g) were dissolved in methanol/water (1:1, v/v; 10 cm^3) under a stream of nitrogen for 10 min. The solution was centrifuged (1400*g*), filtered through glass wool, and applied to a Sephadex LH-20 column (10 g preswollen in 10 cm^3 methanol/water, 1:1, v/v; column dimensions: 10 cm length × 1.5 cm diameter). Nontannin compounds were eluted with 300 cm^3 methanol/water (1:1, v/v), and a tannin fraction was eluted with acetone/water (7:3, v/v; 150 cm^3). Acetone was evaporated in vacuo (35 °C), and then, the aqueous phase was frozen and lyophilized (~24 h). The isolated tannins were stored at −20 °C.

Molecular Weight Characterization of Tannins. Average molecular weights of isolated tannins were determined by gel permeation chromatography (GPC) using a GPC50 instrument with a differential refractive index detector (Polymer Laboratories, Church Stretton, Shropshire, United Kingdom). The tannin samples were dissolved in tetrahydrofuran (THF; 0.2 g tannins in 100 cm^3 THF) at 5 °C overnight. Samples (100 μL) were injected into the GPC system and separated on two serially connected PLgel 3 μm MIXED-E columns (300 mm × 7.5 mm; Polymer Laboratories) and eluted with THF at 1 cm^3 min^{-1} at ambient temperature. Column calibration was performed with polystyrene standards (PSTY EasiVial, Polymer Laboratories). The molecular weight values used were those of the highest peak in chromatograms (M_p). The polydispersity ratio was between 1.3 and 1.5 for the four tannin mixtures studied. Masses were confirmed by MALDI-TOF mass spectrometry analysis of the tannins (*35*). The average molecular weight for the tara gallotannins determined by GPC (593 g mol^{-1}) conflicts with MALDI-TOF mass spectrometry, which suggests that the most abundant molecular masses (*m*/*z*) range from 1432.0 to 1736.2 g mol^{-1}. As discussed later, this means that binding stoichiometries and enthalpies for tara gallotannins should be viewed with caution; however, equilibrium binding constants are not significantly influenced.

ITC. A CSC Nano ITC Series III instrument (Calorimetry Sciences Corp., Lindon, UT) was used to measure enthalpy changes associated with tannin−protein interactions at 298 K. In a typical experiment, buffered gelatin solution (0.002, 0.007, or 0.01 mM) or BSA solution (0.003, 0.015, or 0.075 mM) was placed in the 1.001 cm^3 sample cell of the calorimeter and buffered tannin solution (5 g dm^{-3}) was loaded into the injection syringe. Tannins were titrated into the sample cell as a sequence of 24 injections of 10 μL aliquots. The time delay (to allow equilibration) between successive injections was 3 min. The contents of the sample cell were stirred throughout the experiment at 200 rpm to ensure thorough mixing. Raw data were obtained as a plot of heat (μJ) against injection number and featured a series of peaks for each injection. These raw data peaks were transformed using the instrument software to obtain a plot of observed enthalpy change per mole of injectant (ΔH_{obs}, kJ mol^{-1}) against molar ratio.

Control experiments included the titration of buffered tannin solutions into buffer, buffer into protein, and buffer into buffer; controls were repeated for each buffer system used and at each protein concentration. The last two controls resulted in small and equal enthalpy changes for each successive injection of buffer and, therefore, were not further considered in the data analysis (*33*). Corrected data refer to experimental data after subtraction of the tannin into buffer control data. Tannin molecules tend to self-associate into aggregates due to their hydrophobicity; therefore, when injected from the syringe into buffer, the tannin molecules undergo an endothermic process of deaggregation, analogous to surfactant demicellization (*29*). The extent of deaggregation depends inversely on the concentration of tannin already present in the sample cell; therefore, successive injections of tannin into buffer lead to the observation of progressively lower endothermic enthalpy changes as has been illustrated in earlier work (*29*). The data are shown after subtraction of the effects of tannin deaggregation, which means that the assumption is made that tannins dissociate prior to binding.

Data Analysis. Estimated binding parameters were obtained from the ITC data using the Bindworks ITC data analysis program (Version 3.1.3, Applied Thermodynamics, Hunt Valley, MD). Data fits were obtained using either the independent set of multiple binding sites model or the two sets of multiple binding sites model. For the independent set of multiple binding sites model, the analytical solution for the total heat measured (*Q*) is determined by the formula:

$$Q = V\Delta H\left\{[\mathrm{L}] + \frac{1 + [\mathrm{M}]nK - \sqrt{(1 + [\mathrm{M}]nK - [\mathrm{L}]K)^2 + 4K[\mathrm{L}]}}{2K}\right\}$$

where *V* is the volume of the calorimeter cell, ΔH is enthalpy, [L] is ligand concentration, [M] is macromolecule concentration, *n* is the molar ratio of interacting species, and *K* is the equilibrium binding constant (*36*). The analytical solution for *Q* in the two sets of multiple binding sites model is determined by the formula:

$$Q = V[\mathrm{M}]\left\{\frac{n_1\Delta H_1 \mathrm{K}_1[\mathrm{L}]}{1 + K_1[\mathrm{L}]} + \frac{n_2\Delta H_2 K_2[\mathrm{L}]}{1 + K_2[\mathrm{L}]}\right\}$$

where n_1 and n_2 are the molar ratios of interacting species, ΔH_1 and ΔH_2 are the enthalpies, and K_1 and K_2 are the equilibrium binding constants for each of the two sets of multiple binding sites (*36*). The goodness of fit was determined by calculation of χ^2 from the following formula:

$$\chi^2 = \sum_{i=1}^{N} \frac{[y_i - f(x_i)]^2}{\sigma_i^2}$$

where *N* is the number of data points, y_i is the actual value, $f(x_i)$ is the theoretical value, and σ_i is the measurement error. The data fits were acceptable in each case since the χ^2 values were less than the critical values for the appropriate degree of freedom.

RESULTS

Figure 2 shows the ITC binding isotherms for the interaction of tara, sumac, chestnut, and myrabolan tannins with gelatin as plots of observed changes in enthalpy (ΔH_{obs}) vs tannin:protein molar ratios. Each plot shows an exothermic interaction in which the protein binding sites become completely saturated at tannin:protein molar ratios in excess of 200:1. The high molar ratio values required for saturation suggest multiple binding sites of tannin to protein, and because of these high binding stoichiometries, it was necessary to repeat titrations with different gelatin concentrations present in the calorimeter cell to observe complete binding isotherms; these overlaid data are represented in **Figure 2** by the use of different symbols.

A common feature of each tannin−gelatin binding isotherm was a long region of very small changes in enthalpy at high molar ratios (e.g., greater than 200:1 for tara gallotannins in **Figure 2a**) before zero enthalpies (after correction for dilution enthalpies) were recorded to indicate complete binding saturation. These regions indicate that a secondary and very weak interaction process was occurring at higher molar ratios, and it was therefore necessary to employ a binding model consisting of two sets of multiple binding sites, each with different binding strengths (*36*). The justification for this type of binding model is highlighted in **Figure 3**, which shows fits to the data for the interaction of sumac gallotannins with gelatin using two different binding models: The dashed line assumes one independent set of multiple binding sites with equal binding strength, and the solid line assumes two sets of multiple binding sites with different binding strengths. Inspection of **Figure 3** reveals that the independent set of multiple binding sites model (dashed line) does not describe the data well at low molar ratios (<25:1) or

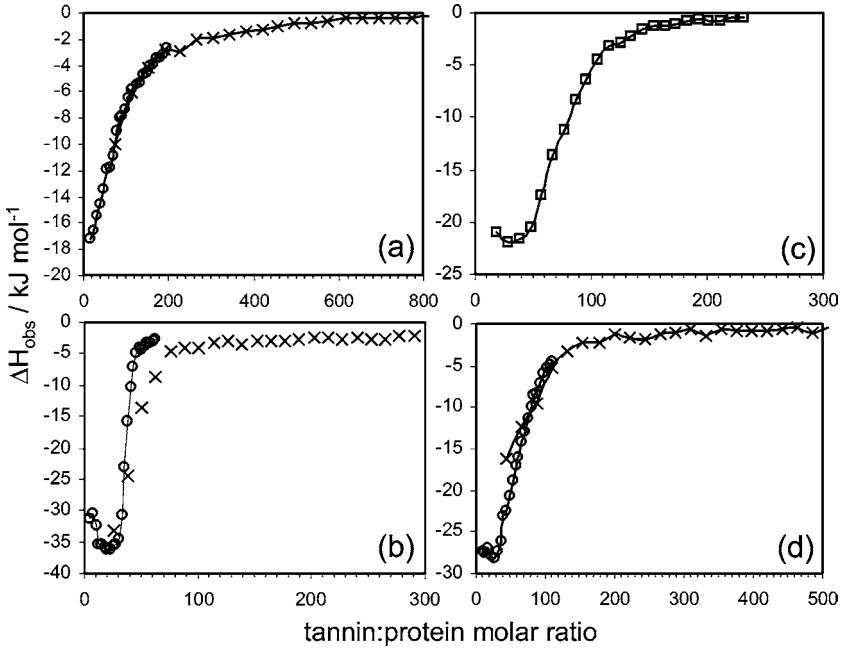

Figure 2. ITC binding isotherms for (**a**) tara gallotannin, (**b**) sumac gallotannin, (**c**) chestnut ellagitannin, and (**d**) myrabolan ellagitannin interactions with gelatin. Symbols denote different gelatin concentrations of 0.01 (circles), 0.007 (squares), and 0.002 mM (crosses).

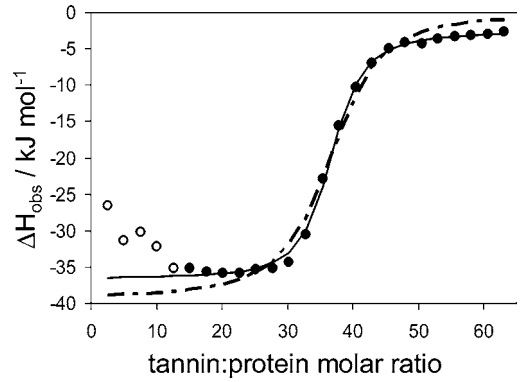

Figure 3. Analysis of sumac gallotannin binding to gelatin. The solid line gives the fit assuming two sets of multiple binding sites with binding parameters as detailed in **Table 1** ($\chi^2 = 2.9$). The dashed line shows the fit assuming one independent set of multiple binding sites where $n = 33$, $K = 2.8 \times 10^5$ M^{-1}, and $\Delta H = -43.5$ kJ mol^{-1} ($\chi^2 > 20.0$). Solid circles represent data used to fit the models. Open circles represent data not included during fitting of binding parameters.

at high molar ratios (≥50:1). Indeed, the slope of the fitted sigmoidal curve is also not an accurate representation of the actual data and the overall fit is poor ($\chi^2 > 20.0$). In contrast, the two sets of multiple binding sites model (solid line) gives a close fit to the data at molar ratios in excess of 15:1 ($\chi^2 = 2.9$). Data at tannin:protein molar ratios <15:1 were not included in the fitting of the binding model depicted in **Figure 3** since these data exhibited increases in exothermicity with subsequent tannin injections to the protein solution. Such behavior at lower tannin:protein molar ratios was also observed for the ellagitannins (**Figure 2c**,**d**) and may be evidence of some cooperative behavior such that tannin binding was enhanced or influenced by prebound tannin molecules (*29*). This feature of the binding isotherms (when present) was not accounted for by the binding models employed.

Because the two sets of the multiple binding sites model was more representative of the data presented in **Figure 2**, it was used to determine the thermodynamic parameters for the tannin−gelatin interactions listed in **Table 1**. The equilibrium binding constants ranged from 8.0×10^3 to 2.2×10^6 M^{-1} for the stronger binding sites (K_1) and from 1.5×10^2 to 1.1×10^4 M^{-1} for the weaker binding sites (K_2). Binding stoichiometries (tannin:protein) were in the range 31:1 to 53:1 for the stronger binding sites (n_1) and 21:1 to 60:1 for the weaker binding sites (n_2).

Figure 4 shows ITC binding isotherms for the tara, sumac, chestnut, and myrabolan tannins to BSA. As with **Figure 2**,

Table 1. Estimated Thermodynamic Binding Parameters for the Interaction of Hydrolyzable Tannins with Gelatin and BSA

	gallotannins		ellagitannins		
	tara	sumac	chestnut	myrabolan	PGG
M_p (g mol^{-1})	593[a]	2069	780	1249	940
			gelatin		
χ^2	6.7	2.9	6.7	2.4	3.9
n_1	53.0	35.4	46.0	36.4	30.8
K_1 (M^{-1})	8.0×10^3	6.9×10^5	1.5×10^6	2.2×10^6	2.8×10^5
ΔH_1 (kJ mol^{-1})	−21.0	−37.6	−22.2	−27.9	−47
n_2	57.6	20.7	32.3	38.8	60
K_2 (M^{-1})	1.5×10^2	4.2×10^2	1.1×10^4	8.3×10^3	7.5×10^2
ΔH_2 (kJ mol^{-1})	−20.9	−49.4	−27	−31.2	−43.8
			BSA		
χ^2	2.0	4.3	9.6	4.2	1.3
n_1	2.5	9.4	17.7	22.0	16.5
K_1 (M^{-1})	1.0×10^4	1.7×10^5	9×10^2	7.0×10^2	2.2×10^5
ΔH_1 (kJ mol^{-1})	−33.0	−30.3	−39.8	−58.1	−37.5
n_2	15.4	24.2			67.1
K_2 (M^{-1})	6.8×10^2	2.2×10^3			6×10^2
ΔH_2 (kJ mol^{-1})	−24.8	−29.7			−48.6

[a] MALDI-TOF mass spectrometry suggests that the most abundant molecular masses (*m*/*z*) range from 1432.0 to 1736.2 g mol^{-1} (*35*). Fitting ITC binding isotherms with a mass in this range (1500 g mol^{-1}) influence binding stoichiometries (e.g., for gelatin, $n_1 = 38.5$, and $n_2 = 22.4$) and enthalpies (e.g., for gelatin, $\Delta H_1 = -46.8$ kJ mol^{-1}, and $\Delta H_2 = -54.0$ kJ mol^{-1}), but equilibrium binding constants remain within the same order of magnitude (e.g., for gelatin, $K_1 = 1.0 \times 10^4$ M^{-1}, and $K_2 = 4.7 \times 10^2$ M^{-1}).

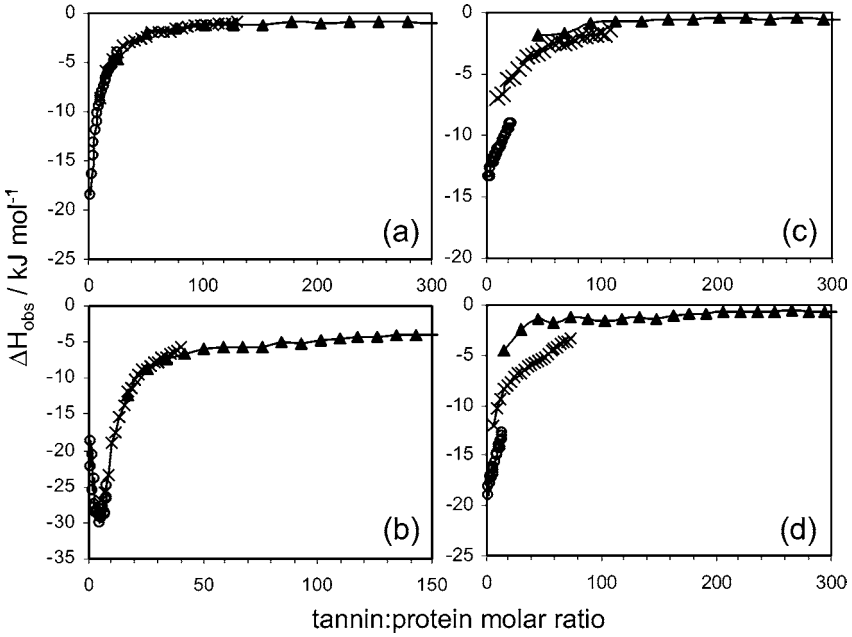

Figure 4. ITC binding isotherms for (**a**) tara gallotannin, (**b**) sumac gallotannin, (**c**) chestnut ellagitannin, and (**d**) myrabolan ellagitannin interactions with BSA. Symbols signify different BSA concentrations of 0.075 (circles), 0.015 (crosses), and 0.003 mM (triangles).

the plots of ΔH_{obs} vs tannin:protein molar ratio feature overlaid data from experiments in which different protein concentrations were present in the calorimeter cell. It can be seen that the shapes of the binding isotherms for the gallotannins (tara and sumac) binding to BSA are similar to those for their binding to gelatin. However, the binding stoichiometries were lower for BSA ($n_1 + n_2 = 17.9-33.6$) than for gelatin ($n_1 + n_2 = 56.1-110.6$). In contrast, the ellagitannins (chestnut and myrabolan) showed a marked difference in their binding to BSA and gelatin. BSA binding isotherms exhibited evidence of protein concentration dependence; that is, at a given tannin:protein molar ratio, the values for ΔH_{obs} are more exothermic at higher BSA concentrations in the calorimeter cell.

As for the tannin−gelatin interaction data, the ITC data for tannin−BSA interactions presented in **Figure 4** were fitted to binding models to allow the determination of thermodynamic parameters as summarized in **Table 1**. In the case of the gallotannin−BSA interactions, the binding models used consisted of two sets of multiple binding sites with different binding strengths as used earlier for tannin−gelatin interactions, and the equilibrium binding constants (K_1 and K_2) were found to be of the same order of magnitude as for gallotannin−gelatin interactions. However, for the ellagitannin−BSA interactions, it was not possible to arrive at a unique fit for all data sets (i.e., at different concentrations) because of the protein concentration dependence. Therefore, for the ellagitannins, the quoted fit in **Table 1** is for the data at 0.015 mM BSA concentration since this was the middle concentration. The data at the other BSA concentrations also were found to fit to a binding model consisting of one set of weak binding sites, giving equilibrium binding constants $<10^3$ M^{-1}. Therefore, the ellagitannins can be regarded to have a weaker interaction with BSA than they do with gelatin.

Figure 5 shows the ITC binding isotherms for the interaction of PGG with gelatin and BSA, and the binding parameters from these data are summarized in **Table 1**. PGG is one of several closely related compounds that occur in sumac gallotannins and was studied to compare the protein interaction of a pure compound with those of tannin mixtures consisting of structurally similar compounds but having a distribution of molecular masses (*35*). PGG is a gallotannin and interacts with gelatin and BSA in a similar fashion as the isolated tara and sumac gallotannins. The PGG data also showed agreement with previously published ITC data on the binding of tannic acid (from which PGG was isolated) to gelatin and BSA (*30*), which also indicated similar binding strengths for these two proteins.

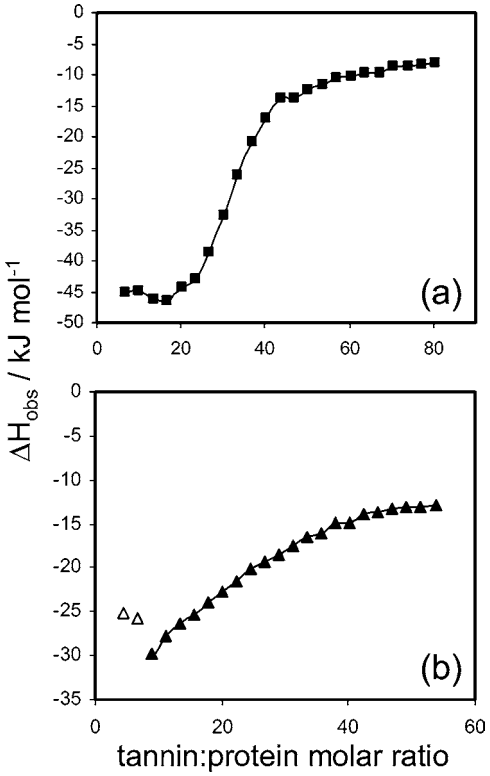

Figure 5. ITC binding isotherms for PGG binding to (**a**) gelatin and (**b**) BSA (open triangles indicate data not used during fitting of binding parameters).

DISCUSSION

It is widely accepted that proteins that have a compact globular tertiary structure (such as BSA) have a poor affinity for tannins, whereas proline-rich proteins (such as gelatin), which have an extended random coil conformation, have a high affinity for tannins (*11*). However, the equilibrium binding constants determined here for the protein binding of gallotannins and ellagitannins suggest that the tertiary structures of tannins are also important; gallotannins bind with equal strength to gelatin and BSA, whereas ellagitannins bind strongly to gelatin and weakly to BSA. On the other hand, there are clear differences in binding stoichiometry; gelatin generally binds more tannin molecules per mole of protein than does BSA, with strong binding sites on gelatin outnumbering those on BSA by a ratio of at least 2:1 in all cases studied here. Therefore, in a competitive binding assay, it would be expected to observe greater relative binding affinity for gelatin than for BSA for all of the hydrolyzable tannins studied here since the binding site stoichiometries favor gelatin binding.

The observed differences in binding characteristics between the gallotannins and the ellagitannins highlight the importance of conformational flexibility of the tannin molecule, as has been discussed in previous literature (*6*, *37*, *38*). The ellagitannins differ from the gallotannins in that the aromatic rings in the hydroxydiphenoyl groups are constrained by intramolecular biphenyl linkages (see **Figure 1**). There is a resulting loss of conformational freedom that appears to play a fundamental role in their protein binding capacity. However, it appears that the loss of conformational freedom in ellagitannins has no major impact on the ability to bind to a flexible protein such as gelatin, whereas it does detrimentally impact the ability to bind to BSA as shown by the low binding constants determined from ITC binding isotherms (**Table 1**). Indeed, this finding is supported by recent work, which demonstrated that PGG (a gallotannin) precipitated BSA more efficiently (i.e., at lower mass ratios) than the ellagitannins castalagin (a component of chestnut ellagitannins) and grandinin (*18*). The ellagitannins studied here also exhibited differences in binding enthalpy with a change in BSA concentration. This type of concentration dependence has been observed previously for the interaction of epicatechin with BSA (*31*) and can be a consequence of ligand-induced protein aggregation (*39*). It is apparent that the conclusion that conformational flexibility of both the tannin and the protein are important complementary factors leading to strong interactions is a key point (*6*). However, from the data presented here, further refinement should be made to state that conformational flexibility of either the tannin or the protein is necessary for strong binding, which then would account for the weaker binding of ellagitannins to BSA where both tannin and protein are more conformationally rigid.

Results for the tara gallotannins suggest that the nature of the polyol core of the hydrolyzable tannins may also have an influence on binding strengths. Tara gallotannins exhibited the weakest interaction with gelatin and uniquely, in addition to the binding model used to generate the binding parameters in **Table 1**, their binding could equally well have been described as an interaction with a single type of weak binding site ($n = 90$, $K = 2.6 \times 10^3$ M^{-1}, $\Delta H = -25.4$ kJ mol^{-1}). Tara gallotannins are distinct from the other tannins studied in that they contain a quinic acid core as opposed to glucose (see **Figure 1**). However, MALDI-TOF mass spectrometry suggests that tara gallotannins also contain only one elongated chain in their structure (*35*), whereas sumac gallotannins may have several elongated chains; therefore, an alternative explanation could be related to the relative abilities to act as multidentate ligands.

While the importance of tannin structure is recognized in the literature, emphasis has also been given to considerations of molecular size and water solubility (or polarity) (*6*, *37*, *38*). The relative importance of these other parameters is shown here to be low. For example, the water solubilities indicated by the octanol/water partition coefficients (K_{ow} values) of sumac gallotannins and myrabolan ellagitannins were found to be similar ($K_{ow} = 1.96$ and 1.49, respectively; unpublished data); yet, each demonstrated radically different protein binding behavior. Myrabolan ellagitannins exhibited an apparent 4 orders of magnitude greater binding strength for gelatin than for BSA, while sumac gallotannins had similar binding strengths for gelatin and BSA.

It has been suggested that the interaction of proteins with tannins is primarily a surface phenomenon and that tannin molecules effectively coat the surface of a protein (*37*). Studies of the stoichiometry of tannin−protein precipitates have supported this view with observations of very high stoichiometries that indicate that binding is unlikely to be to specific ligand binding sites on the protein (*19*, *40*, *41*). The data from ITC also suggest that tannins could bind by coating the protein surface. It was previously estimated that the maximum amount of PGG, which could be bound on the surface of BSA, would be of the order of 100 mol PGG/mol BSA (*40*); the binding stoichiometry found here indicated binding of ~84 mol PGG/mol BSA. However, it is noteworthy that two classes of binding sites exist on both gelatin and BSA, which suggests that there is some specificity for particular sites (e.g., hydrophobic residues).

As a final point, it was found that the ITC binding isotherms observed for myrabolan ellagitannins in this study were markedly different to those reported in earlier work (*29*). In the earlier study, "myrabolan tannins" consisted of the crude commercially available product as supplied to the leather industry, which also contains nontannin impurities. In the present study, chromatography on Sephadex LH20 was used to separate tannins from nontannin impurities, yielding 0.145 g tannins/g air-dried commercial product. It was noted previously that for the crude "myrabolan tannin product" there was concentration dependence for the interaction with gelatin and BSA, whereas in the present study on the isolated tannin fraction only the interaction with BSA showed concentration dependence and equivalent binding enthalpies to the previous data (*29*). However, in comparison to the data reported here for the isolated myrabolan ellagitannins, binding of the crude tannin product to gelatin resulted in binding isotherms with significantly lower binding enthalpies. Potentially, some of the impurities in the crude tannin products could be bound to the tannins and thus may have inhibited binding to proteins. This might have important implications for the extrapolation of data from pure systems to the complexities of the real in vivo scenario in which a range of other substances can be present (*42*).

ABBREVIATIONS USED

BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; GPC, gel permeation chromatography; HPLC, high-performance liquid chromatography; ITC, isothermal titration calorimetry; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; NMR, nuclear magnetic resonance; PGG, pentagalloyl glucose; THF, tetrahydrofuran.

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