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Probing Protein−Tannin Interactions by Isothermal Titration Microcalorimetry

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Isothermal titration microcalorimetry (ITC) has been applied to investigate protein−tannin interactions. Two hydrolyzable tannins were studied, namely myrabolan and tara tannins, for their interaction with bovine serum albumin (BSA), a model globular protein, and gelatin, a model proline-rich random coil protein. Calorimetry data indicate that protein−tannin interaction mechanisms are dependent upon the nature of the protein involved. Tannins apparently interact nonspecifically with the globular BSA, leading to binding saturation at estimated tannin/BSA molar ratios of 48:1 for tara- and 178:1 for myrabolan tannins. Tannins bind to the random coil protein gelatin by a two-stage mechanism. The energetics of the first stage show evidence for cooperative binding of tannins to the protein, while the second stage indicates gradual saturation of binding sites as observed for interaction with BSA. The structure and flexibility of the tannins themselves alters the stoichiometry of the interaction, but does not appear to have any significant affect on the overall binding mechanism observed. This study demonstrates the potential of ITC for providing an insight into the nature of protein−tannin interactions.

KEYWORDS: Tannin; polyphenol; protein; albumin; gelatin; titration microcalorimetry

INTRODUCTION

Despite numerous studies, the nutritional implications of tannins (plant polyphenols) for humans and animals are poorly understood (*1*, *2*). Tannins form soluble and/or insoluble complexes with proteins (*3*), which are thought to account for the generally poor amino acid absorption from tannin-rich feeds by monogastric animals (*4*). However, in ruminants, the literature is contradictory on the role of tannins, because they can reduce or enhance amino acid absorption, and it is not yet known what properties of tannins define these opposite nutritional effects (*5*). Consequently, it is questionable whether it is appropriate to determine the total quantity of tannins to predict the nutritional value of plants (*6*). In humans, tannins have drawn attention for their beneficial health effects (e.g., antioxidant capacity) (*2*). However, their propensity for binding to proteins may impact upon their bioavailability (*7*) and/or mask their antioxidant capacity in vivo (*8*, *9*). There is also evidence that some tannins can be toxic to humans, monogastric animals, and ruminants (*2*, *5*), although animal studies demonstrate that in vitro toxicity is often not observed in vivo (*2*).

Currently, there are no techniques that reliably relate in vitro to in vivo effects of tannins (*10*), particularly to clarify the role of protein−tannin interactions. Nonetheless, from the existing literature, we can conclude that tannins are *not* universal protein binding agents (*11*) and that protein−tannin binding affinities can vary as much as 10,000-fold (*12*). Structure−activity relationships therefore would appear to be important to protein−tannin interactions (*11*−*14*). Tannins tend to have poor affinity for small, tightly folded globular proteins, whereas it has been reported that tannins bind preferentially to proline-rich proteins (PRPs) that have either random-coil or collagen-like helical conformations (*11*, *12*). Tannins also have a strong affinity for histidine-rich histatins (*15*). Results from X-ray crystallography and NMR spectroscopy suggest that the protein−tannin interaction is driven initially by hydrophobic effects and that complexes are stabilized by hydrogen bonding (*16*, *17*). Tannins may also act as multidentate ligands to facilitate protein cross-linking and consequent precipitation (*18*, *19*). This has led to the inference that high molecular weight (MW) tannin molecules should precipitate proteins more effectively (*17*); although, recent data suggests this rule is an oversimplification and does not apply to all tannins (*10*). Indeed, Bacon and Rhodes found that, while

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the higher MW molecules present in tara tannins had higher affinity for PRPs than the lower MW tetra- and penta-galloylglucose, the smaller tetra-galloylglucose had higher affinity than the larger penta-galloylglucose (*20*). Similarly, it has been reported that structurally homologous ellagitannins can exhibit remarkably different binding properties despite similar MW (*21*). These represent a few examples of the many contradictory results relating to the study of protein−tannin interactions.

In the present study, isothermal titration microcalorimetry (ITC) has been employed to characterize the binding of two contrasting tannin types, flexible gallotannins (tara tannins) and more rigid gallo- and ellagitannins (myrabolan tannins), to two model proteins, bovine serum albumin (BSA) and gelatin. BSA is a globular protein and has been used previously as a model for Rubisco, the major protein in green plants (*6*, *10*). Gelatin is proline-rich, has an open random coil conformation, and is a model for seed prolamines and salivary PRPs, the latter being the focus of recent research into the molecular basis for astringency (*14*, *20*, *22*). The two tannin samples studied were the natural mixtures of extracted tannins rather than purified single compounds, and as such, represented more accurately those tannins that occur naturally in foods and animal feeds. ITC is a powerful technique for the study of the thermodynamics of protein−ligand interactions and is discussed in detail elsewhere (*23*−*26*). 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 (*26*). Indeed, the technique is particularly attractive because 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. Our objectives in this study were to explore the potential of ITC to determine differences in the nature of protein−tannin interactions by probing whether tannins bind to conformationally different proteins in the same way and whether tannin structure influences the nature of binding.

MATERIALS AND METHODS

Materials. Myrabolan (ex India) and tara (ex Peru) tannins were obtained from Forestal Quebracho Ltd. (Reading, U.K.) and were prepared as 5 g dm^{-3} solutions. BSA (purity ≥ 99%, essentially globulin free) and bovine skin gelatin were purchased from Sigma (Poole, Dorset, U.K.). All solutions were prepared in 50 mM acetate buffer at pH 5 and were degassed prior to use.

Microcalorimetry. An Omega ITC instrument (MicroCal, Northampton, MA) was used to measure enthalpy changes associated with protein−tannin interactions at 298 K. In a typical experiment, protein solution was placed in the 1.4115-cm^3 sample cell of the calorimeter and tannin solution was loaded into the injection syringe. Tannin solution was titrated into the sample cell as a sequence of 50 injections of 5×10^{-6} dm^3 aliquots. The duration of each injection was 5 s, and the time delay (to allow equilibration) between successive injections was 3 min. The contents of the sample cell were stirred throughout the experiment at 400 rpm to ensure thorough mixing. Raw data were obtained as a plot of heat flow (μcal s^{-1}) against time (min) as shown in **Figure 1**. These raw data were then integrated peak-by-peak and normalized to obtain a plot of observed enthalpy change per mole of injectant (ΔH_{obs}, kJ mol^{-1}) against tannin concentration (g dm^{-3}) or weight ratio (tannin/protein). Negative ΔH_{obs} represents an exothermic change, and positive ΔH_{obs} represents an endothermic change. Peak-by-peak integration was performed using Microcal Origin (Microcal Software, Northampton, MA), whereas normalization and extrapolation of calorimetry data were performed using Micosoft Excel. For the purposes of normalizing data, the average molecular weights were 650 g mol^{-1} for myrabolan tannins and 1500 g mol^{-1} for tara tannins.

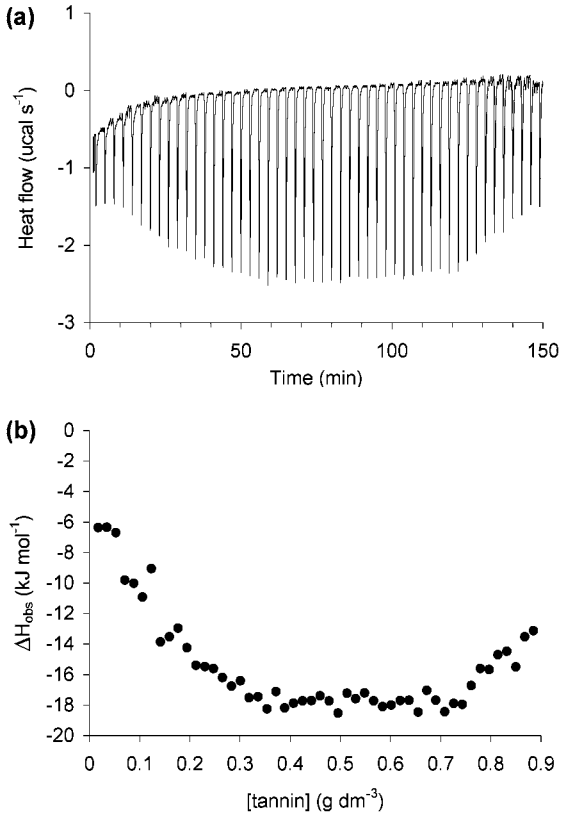

Figure 1. (a) Typical raw data plot of heat flow against time for the titration of tara tannins into 1.0 g dm^{-3} gelatin. Each peak corresponds to the heat change associated with injection of a 5×10^{-6} dm^3 aliquot of tannin solution into the calorimeter cell containing the gelatin solution. A negative heat flow represents an exothermic change, and a positive heat flow represents an endothermic change. (b) Corresponding plot after integration of peak areas and normalization with respect to injectant concentration to yield a plot of molar enthalpy change (ΔH_{obs}) against myrabolan tannin concentration.

Control experiments included the titration of tannin into buffer, buffer into protein, and buffer into buffer. The corrected experimental data have had the corresponding tannin into buffer and buffer into protein controls subtracted. The buffer into buffer control is added to the data because buffer dilution is an integral part of the other controls and has thus been subtracted twice (*26*). The titration of tannin into buffer yielded enthalpy changes that are discussed later.

RESULTS

Interaction of Myrabolan Tannins with BSA. The interaction of myrabolan tannins (5 g dm^{-3}) with BSA was studied at three different concentrations of the protein, namely 0.2, 1.0, and 5.0 g dm^{-3}. The calorimetry data for these three experiments and the control titration of myrabolan tannins into buffer are plotted as observed molar enthalpy change (ΔH_{obs}) against tannin concentration in **Figure 2a**. For the interaction with BSA, the titration plots show that each injection of myrabolan tannins is accompanied by an exothermic enthalpy change (i.e., heat is released). A marked effect of BSA concentration on the magnitude of ΔH_{obs} is observed, although the general shapes of the plots are similar. The titration plot at the lowest concentration of BSA (0.2 g dm^{-3}) runs closely parallel with the control (difference < 0.6 kJ mol^{-1}) at added tannin concentrations in excess of 0.35 g dm^{-3}. This suggests that saturated binding conditions have been reached.

Usually, the control experiment for injection of a ligand into buffer will consist of a series of equal heats of dilution (*26*). In

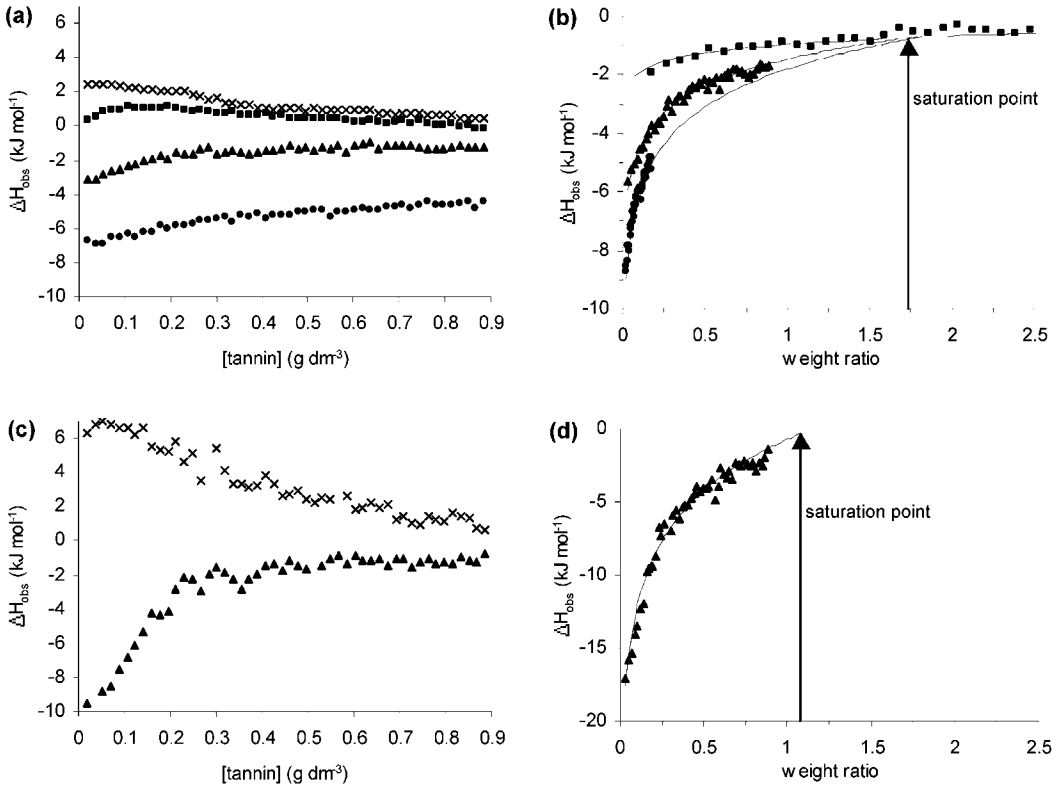

Figure 2. (a) Plots of molar enthalpy change (ΔH_{obs}) against myrabolan tannin concentration for the interaction with BSA. (b) Binding isotherms extrapolated to saturation (0.2 g dm^{-3} BSA, $y = 0.41 \cdot \mathrm{Ln}(x) - 0.96$, $R^2 = 0.84$; 1.0 g dm^{-3} BSA, $y = 1.33 \cdot \mathrm{Ln}(x) - 1.47$, $R^2 = 0.98$; 5.0 g dm^{-3} BSA, $y = 1.85 \cdot \mathrm{Ln}(x) - 1.81$, $R^2 = 0.98$) showing ΔH_{obs} against weight ratio (myrabolan tannin/BSA). (c) Plot of molar enthalpy change (ΔH_{obs}) against tara tannin concentration for the interaction with BSA. (d) Extrapolated binding isotherm ($y = 5.03 \cdot \mathrm{Ln}(x) - 0.72$, $R^2 = 0.89$) showing ΔH_{obs} against weight ratio (tara tannin/BSA). (×) buffer; (■) 0.2 g dm^{-3} BSA; (▲) 1.0 g dm^{-3} BSA; (●) 5.0 g dm^{-3} BSA.

this case, the myrabolan tannins are injected from a concentrated solution in which the tannin molecules will tend to self-associate into aggregrates due to their hydrophobicity (*22*, *27*). Therefore, when injected into buffer, the tannin molecules will undergo an endothermic (i.e., heat absorption) process of deaggregation, analogous to data for surfactant demicellization (*28*). The extent of deaggregation will depend inversely on the concentration of tannin already present in the sample cell, because the tannin concentration in the cell will eventually exceed the critical aggregation concentration (cf. surfactant critical micelle concentration) and subsequent enthalpy changes will correspond to dilution of tannin aggregates. Therefore, further injections will lead to the observation of progressively lower endothermic ΔH_{obs}. Unfortunately, it is not possible to determine a critical aggregation concentration from the calorimetry data, because this value usually depends on molecular weight, and the present study used a mixture of myrabolan tannins with molecular weights ranging from 332 to 1084 g mol^{-1}.

The control corrected calorimetry data are plotted in **Figure 2b** against the weight ratio of myrabolan tannins to BSA in the sample cell. By correcting the data to take into account the effects of tannin dilution, the assumption is made that tannins dissociate prior to binding. This representation of the data clearly shows the dependence of the interaction upon BSA concentration. At higher BSA concentration, the initial ΔH_{obs} are of greater magnitude, and the slopes of the plots are also steeper. The values for ΔH_{obs} measured at a given weight ratio do not linearly relate to BSA concentration. However, the binding isotherms in **Figure 2b** approximate to logarithmic curves that can be extrapolated to intersect at a weight ratio of 1.75, which converts to an approximate tannin/BSA molar ratio of ∼178:1 (assuming average molecular weights of 650 g mol^{-1} for myrabolan tannins and 66 000 g mol^{-1} for BSA). This weight ratio corresponds to the apparent saturation point observed in the 0.2 g dm^{-3} BSA data in **Figure 2a**, which implies that binding saturation is indeed reached.

Interaction of Myrabolan Tannins with Gelatin. The interaction of myrabolan tannins (5 g dm^{-3}) with gelatin was also studied at three different protein concentrations, namely 0.1, 0.2, and 1.0 g dm^{-3}. The calorimetry data for these three experiments is plotted in **Figure 3a**, alongside the control where myrabolan tannins were titrated into buffer. As was the case for BSA, the titration plots show a similar general shape to each other, with larger exothermic ΔH_{obs} measured at higher gelatin concentration. However, the general shape of the plots is radically different from that observed for BSA. The data show evidence of two stages of interaction; the first stage shows increasing exothermic ΔH_{obs} with each injection of tannin, and the second stage shows a decrease in the magnitude of ΔH_{obs} with each successive injection (myrabolan tannins and BSA only interact via this second stage). The two stages of interaction are more pronounced when the data are plotted with the controls subtracted against weight ratio in **Figure 3b**. While the transition between stages (determined by inspection as the minimum in each plot) occurs at approximately the same weight ratio when the gelatin concentration is 0.1 and 0.2 g dm^{-3}, the weight ratio is lower for this transition at the higher concentration of 1 g dm^{-3}. As for the BSA data, the second stages of the gelatin curves were extrapolated using a logarithmic function at the lower gelatin concentrations of 0.1 and 0.2 g dm^{-3}. Such extrapolation was not possible at the highest gelatin concentration, since the quality of the fit was very poor ($R^2 < 0.1$).

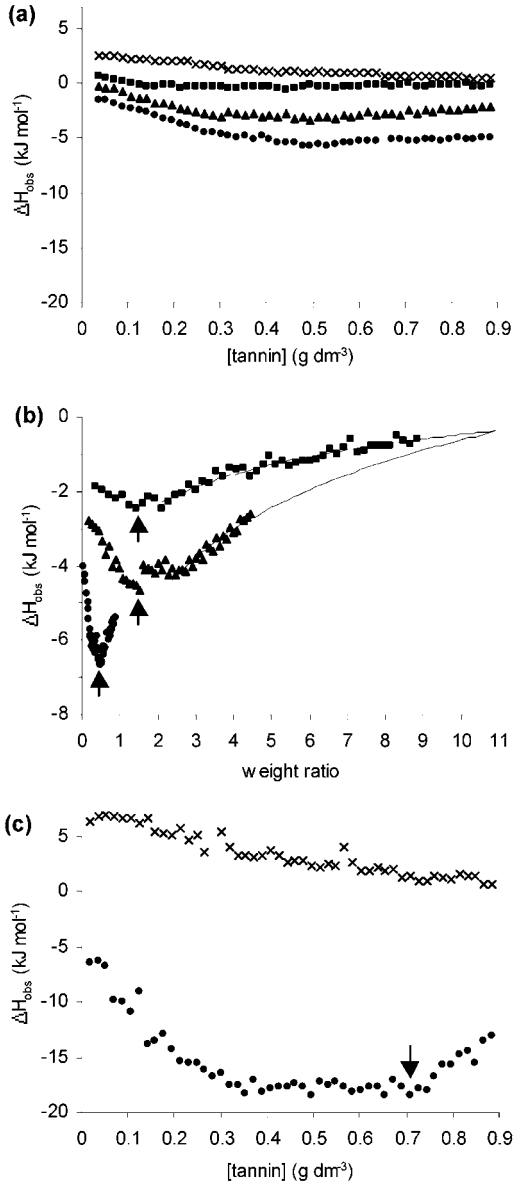

Figure 3. (a) Plots of molar enthalpy change (ΔH_{obs}) against myrabolan tannin concentration for the interaction with gelatin. (b) Binding isotherms, with second parts of curves extrapolated at lower gelatin concentrations (0.1 g dm^{-3} gelatin, $y = 1.18 \cdot \mathrm{Ln}(x) - 3.17$, $R^2 = 0.94$; 0.2 g dm^{-3} gelatin, $y = 2.62 \cdot \mathrm{Ln}(x) - 6.66$, $R^2 = 0.92$), showing ΔH_{obs} against weight ratio (myrabolan tannin:gelatin). Arrows indicate the transition point between the first and second stages of the interaction. (c) Plot of molar enthalpy change (ΔH_{obs}) against tara tannin concentration for the interaction with gelatin. The arrow indicates an inflection in the tannin–gelatin binding isotherm at a tannin/gelatin weight ratio of 0.7. (×) buffer; (■) 0.1 g dm^{-3} gelatin; (▲) 0.2 g dm^{-3} gelatin; (●) 1.0 g dm^{-3} gelatin.

Extrapolation led to intersection of the data at a tannin/gelatin weight ratio of approximately 11:1, which may indicate binding saturation.

Interaction of Tara Tannins with Proteins. The calorimetry data for the interaction of tara tannins with BSA and gelatin are shown in **Figures 2c**,**d** and **Figure 3c**, alongside the corresponding data for myrabolan tannins. The general appearance of the tara tannins data is similar to that observed for the myrabolan tannins, both in terms of the control experiment and binding to proteins. However, direct comparison of the data for tara and myrabolan tannins at the same concentration of each protein reveals differences in the magnitude of ΔH_{obs} and the slopes of the plots. In the case of BSA (**Figure 2**), the tara tannins initially give rise to approximately 3-fold larger exothermic values for ΔH_{obs}, yet by the end of the experiment, any difference in ΔH_{obs} relative to myrabolan tannins is negligible. This yields a noticeably steeper binding isotherm and the appearance that apparent saturation would be reached at a lower tannin/protein weight ratio. Indeed, by fitting a logarithmic curve to the tara tannins data as in **Figure 2d**, the extrapolated saturation point appears at a weight ratio of approximately 1.1, corresponding to a molar ratio of ~48:1 (assuming an average molecular weight of 1500 g mol^{-1} for tara tannins). For gelatin, it is notable that the tara tannin binding isotherm has an inflection at a tannin/protein weight ratio of 0.7 (see **Figure 3c**), whereas such an inflection is absent from the myrabolan tannins data.

DISCUSSION

Calorimetric isotherms for protein−ligand interactions must be interpreted carefully, because ΔH_{obs} will represent the total enthalpy change for the process(es) occurring in the sample cell. In the case of the protein−tannin systems, changes in enthalpy are likely to arise from a combination of binding and aggregation, because these are the two commonly observed features of protein−tannin binding (*17*). Indeed, the observed dependence of ΔH_{obs} on protein concentration is consistent with ligand-induced changes in the protein aggregation state (*30*).

It is clear from the calorimetry data for tannin interactions with BSA and gelatin that protein structure fundamentally influences the observed interaction mechanism. BSA is a globular protein that is low in proline (<5 proline residue %) (*29*), and its calorimetry data indicate a single-stage interaction mechanism. Gelatin, on the other hand, has an open random coil structure and is rich in proline residues (~24 proline/hydroxyproline residue %) (*17*). Its interaction with tannins has two discrete stages with respect to titrated tannins that can be clearly identified from the calorimetry data.

BSA−Tannin Interactions. Overall consideration of the general shape of the binding isotherms encourages the hypothesis that tannins interact nonspecifically with BSA rather than binding to specific receptor sites on the protein. This is because the binding isotherms do not exhibit the sigmoidal shape expected for a specific interaction of a ligand with a protein receptor site or combination of sites (*23*, *26*). The most compelling parallel to the shape of the BSA−tannin isotherms is with those exhibited for surfactant interactions with hydrophobically modified polymers (*31*). The binding isotherms for these nonspecific hydrophobic interactions show a gradual decrease of exothermicity as surfactant is titrated into the polymer solution that is similar to the BSA−tannin isotherms. Moreover, increasing the polymer concentration has the same affect as observed for increasing the BSA concentration here (i.e., exothermicity of the interaction is increased, while saturation occurs at the same ratio of interacting species). Similarly shaped binding isotherms have also been observed for the binding of nonionic surfactants to BSA, although no explanation for the shape of the curves could be resolved (*32*).

The apparently high number of tannin molecules bound per BSA molecule, which is in the approximate range of 48−178 for the tannins studied here, also does not suggest a specific interaction, although it also does not preclude a specific interaction followed by an aggregation process. Interestingly, earlier studies of BSA−tannin binding have noted stoichiometries in this range after analysis of precipitates (*33*, *34*).

However, these studies further imply that the stoichiometry of precipitates is dependent on protein concentration (*33*). This does not hold with our observation of a common extrapolated saturation point for the myrabolan tannins. This anomaly may indicate that saturation of binding sites is not necessary for precipitation to occur. Alternatively, injected tannins may cooperatively associate by hydrophobic interaction with protein-bound tannins rather than binding to different sites on the protein. This would affect the observed stoichiometry of protein−tannin aggregates.

Gelatin−Tannin Interactions. The data for the interaction of tannins with gelatin indicate a different mechanism than with BSA. It is apparent from the data for both myrabolan and tara tannins that there are at least two discrete stages to the interaction (**Figure 3**). The first stages of the binding isotherms (**Figure 3b**) are consistent with cooperative (or synergistic) binding of the tannin to gelatin, which is indicated by the exothermic increase in ΔH_{obs} for each successive injection. This observation implies that the tannins already titrated into the sample cell, and presumably bound to gelatin, have an effect on the new binding interactions. This participation is an example of positive cooperativity and is in agreement with the cooperative effects in PRP−tannin binding observed by others (*22*). Such effects appear to be absent from the BSA−tannin interactions, which indicates a fundamental difference in the nature of the observed binding mechanism.

Interestingly, for the two lower concentrations of gelatin (0.1 and 0.2 g dm^{-3}, **Figure 3b**), the peak exothermicity (i.e., the most negative value for ΔH_{obs}) is reached at approximately the same tannin/gelatin weight ratio (1.5 or 3:2). This may imply that a transition point has been reached. Following the three-stage model for tannin binding and precipitation by PRPs proposed by Charlton et al. (*22*), it could be argued that the peak exothermicity indicates the tannin concentration at which saturation of specific binding sites has been reached. This would suggest that, unlike BSA−tannin, gelatin−tannin interactions initially occur between the tannins and specific sites on the protein. At the highest gelatin concentration, the apparent transition point lies at a 3-fold lower tannin/gelatin weight ratio (0.5 or 1:2), which could be accounted for by the multidentate tannin ligands forming intermolecular cross-links between binding sites on adjacent gelatin molecules in this more concentrated regime. This would follow the general observation that less tannin is required to precipitate proteins from concentrated solutions than from dilute solutions (*17*).

The second stage in the interaction commences after the peak exothermicity is reached in the binding isotherm (**Figure 3b**). After this peak, ΔH_{obs} becomes progressively less exothermic with each injection of tannin. Indeed, the manner of the decrease in exothermicity appears to follow a similar trend to that observed in the BSA−tannin systems and could similarly be fitted to a logarithmic function at the lower gelatin concentrations. This leads to the suggestion that ΔH_{obs} during this second stage is dominated by less specific binding and aggregation.

Myrabolan versus Tara Tannins. The discussion above has concentrated on the interactions of myrabolan tannins with BSA and gelatin. As shown in **Figures 2** and **3**, tara tannins exhibit similar binding isotherms as myrabolan tannins for BSA and gelatin, respectively. However, some differences are apparent from direct comparison of the calorimetry data for myrabolan and tara tannins. The most immediate difference is in the considerably larger exothermic ΔH_{obs} values for tara tannins, indicating a stronger interaction.

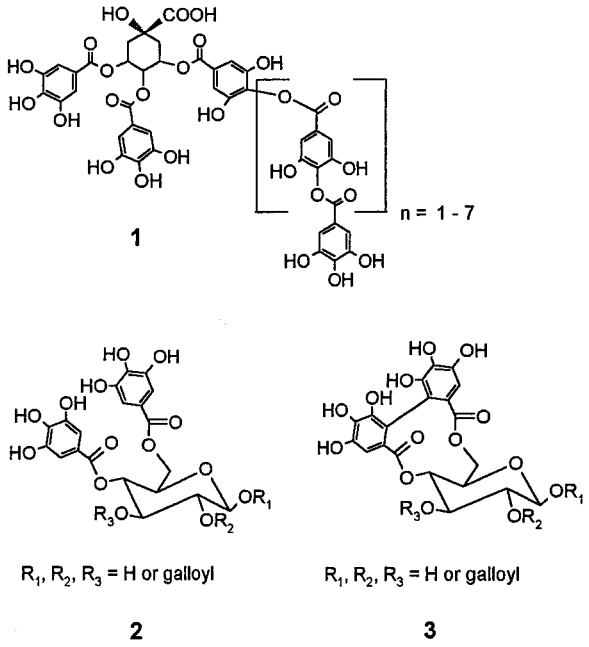

Figure 4. Examples of structures for tara tannins (**1**) and myrabolan tannins (**2** and **3**) that illustrate the range of oligomeric mixtures of gallotannins (**1** and **2**) and ellagitannins (**3**).

For binding to BSA (**Figure 2c**), it is clear that the binding sites on BSA are saturated at a lower tannin concentration by the tara tannins. Extrapolation of the isotherm in **Figure 2d** using a logarithmic function indicates apparent saturation at a weight ratio of 1.1, translating to a tannin/BSA molar ratio of ~48:1 for tara tannins. Despite the error inherent in the extrapolation ($R^2 = 0.89$), it is clear that the saturation point occurs at lower tara tannins concentration than that for myrabolan tannins (~178:1). This observation suggests stronger affinity of the tara tannins for BSA. MALDI-ToF mass spectrometry of tara tannins revealed that the central quinic acid core is surrounded by up to 13 galloyl groups, which means that several gallic acids are depsidically linked and form one or more flexible chains (see structure 1 in **Figure 4**) (*35*, *36*). Presumably the greater conformational flexibility of the tara tannins enhances protein−tannin binding (*17*). Myrabolan tannins are more rigid molecules that do not feature the flexible chains of depsidically linked gallic acids (*35*, *37*). MALDI-ToF mass spectrometry of the myrabolan tannins sample used here (unpublished data) confirmed that they consist of a glucose core surrounded by 1−5 galloyl groups, plus a homologous series of ellagitannins as depicted in structures 2 and 3 in **Figure 4** (*35*).

In the case of binding to gelatin, the obvious difference in **Figure 3c** is that tara tannins have a more exothermic interaction with gelatin than do myrabolan tannins. Also, the second stage of the binding isotherm is steeper, particularly after a weight ratio of 0.7 is reached, which possibly indicates that aggregation occurs more significantly at a lower tannin concentration. Again, as for binding to BSA, this could be attributed to the greater conformational flexibility of tara tannins. Perhaps this flexibility allows the tara tannin molecules to act more efficiently as multidentate ligands and cross-linkers.

To summarize, the structure and flexibility of the tannins alters the energetics and stoichiometry of the interaction, but does not appear to have any effect on the overall binding mechanism observed. This is primarily determined by the nature of the protein involved. Our results have shown that by use of ITC it is possible to derive meaningful data for the interaction of tannin mixtures with different proteins. Future work will need

to investigate a wider range of protein and tannin structures to address several outstanding issues, such as whether binding mechanisms are related to protein digestibility. In addition, it will be important to study purified molecules to answer why some closely related tannin compounds exhibit very different binding specificity.

ABBREVIATIONS USED

BSA, bovine serum albumin; ITC, isothermal titration microcalorimetry; MW, molecular weight; PRP, proline-rich protein.

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LITERATURE CITED

(1) Mueller-Harvey, I.; McAllan, A. B. Tannins − their biochemistry and nutritional properties. In *Advances in Plant Cell Biochemistry and Biotechnology*; Morrison, I. M., Ed.; JAI Press: London, 1992; Vol. 1, pp 151−217.

(2) Duthie, G. G.; Duthie, S. J.; Kyle, J. A. M. Plant polyphenols in cancer and heart disease: implications as nutritional antioxidants. *Nutr. Res. Rev.* **2000**, *13*, 79−106.

(3) Hagerman, A. E. Tannin-protein interactions. In *Phenolic Compounds in Food and Their Effects on Health I. Analysis, Occurrence, and Chemistry*; Ho, C. T.; Lee, C. Y.; Huang, M. T., Eds.; American Chemical Society: Washington, D. C., 1992; ACS Symposium Series 506, pp 236−247.

(4) Lowry, J. B.; McSweeney, C. S.; Palmer, B. Changing perceptions of the effect of plant phenolics on nutrient supply in the ruminant. *Aust. J. Agric. Res.* **1996**, *47*, 829−842.

(5) Caygill, J. C.; Mueller-Harvey, I. *Secondary Plant Products − Antinutritional and Beneficial Actions in Animal Feeding*; Nottingham University Press: Nottingham, U.K., 1999; 129 pp.

(6) Martin, J. S.; Martin, M. M. Tannin assays in ecological studies. Precipitation of ribulose-1,5-bisphosphate carboxylase/oxygenase by tannic acid, quebracho, and oak foliage extracts. *J. Chem. Ecol.* **1983**, *9*, 285−294.

(7) Serafini, M.; Ghiselli, A.; Ferro-Luzzi, A. *In vivo* antioxidant effect of green and black tea in man. *Eur. J. Clin. Nutr.* **1996**, *50*, 28−32.

(8) Riedl, K. M.; Hagerman, A. E. Tannin-protein complexes as radical scavengers and radical sinks. *J. Agric. Food Chem.* **2001**, *49*, 4917−4923.

(9) Arts, M. J. T. J.; Haenen, G. R. M. M.; Wilms, L. C.; Beetstra, S. A. J. N.; Heijnen, C. G. M.; Voss, H.-P.; Bast, A. Interactions between flavonoids and proteins: effect on the total antioxidant capacity. *J. Agric. Food Chem.* **2002**, *50*, 1184−1187.

(10) Osborne, N. J. T.; McNeill, D. M. Characterisation of Leucaena condensed tannins by size and protein precipitation capacity. *J. Sci. Food Agric.* **2001**, *81*, 1113−1119.

(11) Asquith, T. N.; Butler, L. G. Interactions of condensed tannins with selected proteins. *Phytochemistry* **1986**, *25*, 1591−1593.

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

(13) Hagerman, A. E.; Rice, M. E.; Ritchard, N. T. Mechanisms of protein precipitation for two tannins, pentagalloyl glucose and epicatechin$_{16}$ (4→8) catechin (procyanidin). *J. Agric. Food Chem.* **1998**, *46*, 2590−2595.

(14) de Freitas, V.; Mateus, N. Structural features of procyanidin interactions with salivary proteins. *J. Agric. Food Chem.* **2001**, *49*, 940−945.

(15) Yan, Q. Y.; Bennick, A. Identification of histatins as tannin-binding protein in human saliva. *Biochem. J.* **1995**, *311*, 341−347.

(16) Oh, H. I.; Hoff, J. E.; Armstrong, G. S.; Haff, L. A. Hydrophobic interaction in tannin-protein complexes. *J. Agric. Food Chem.* **1980**, *28*, 394−398.

(17) Haslam, E. Natural polyphenols (vegetable tannins) as drugs: possible modes of action. *J. Nat. Prod.* **1996**, *59*, 205−215.

(18) 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.* **1985**, *2*, 1429−1438.

(19) 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 analogues with bovine serum albumin: ramifications for polyphenol-protein molecular recognition. *Phytochemistry* **1999**, *51*, 867−872.

(20) Bacon, J. R.; Rhodes, M. J. C. Development of a competition assay for the evaluation of the binding of human parotid salivary proteins to dietary complex polyphenols and tannins using a peroxidase-labeled tannin. *J. Agric. Food Chem.* **1998**, *46*, 5083−5088.

(21) Zhu, M.; Phillipson, J. D.; Greengrass, P. M.; Bowery, N. E.; Cai, Y. Plant polyphenols: biologically active molecules or nonselective binders to protein? *Phytochemistry* **1997**, *44*, 441−447.

(22) 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.

(23) Wiseman, T.; Williston, S.; Brandts, J. F.; Lin, L.-N. Rapid measurement of binding constants and heats of binding using a new titration calorimeter. *Anal. Biochem.* **1989**, *179*, 131−137.

(24) Blandamer, M. J.; Cullis, P. M.; Engberts, J. B. F. N. Titration microcalorimetry. *J. Chem. Soc., Faraday Trans.* **1998**, *94*, 2261−2269.

(25) Jelesarov, I.; Bosshard, H. R. Isothermal titration calorimetry and differential scanning calorimetry as complementary tools to investigate the energetics of biomolecular recognition. *J. Mol. Recognit.* **1999**, *12*, 3−18.

(26) O'Brien, R.; Ladbury, J. E.; Chowdry, B. Z. Isothermal titration calorimetry of biomolecules. In *Protein−Ligand Interactions: Hydrodynamics and Calorimetry*; Harding, S. E., Chowdry, B. Z., Eds.; Oxford University Press: Oxford, U.K., 2001; pp 263−286.

(27) Saucier, C.; Bourgeois, G.; Vitry, C.; Roux, D.; Glories, Y. Characterization of (+)-catechin-acetaldehyde polymers: a model for colloidal state of wine polyphenols. *J. Agric. Food Chem.* **1997**, *45*, 1045−1049.

(28) Engberts, J. B. F. N.; Blandamer, M. J. Understanding organic reactions in water: from hydrophobic encounters to surfactant aggregates. *Chem. Commun.* **2001**, *18*, 1701−1708.

(29) Hirayama, K.; Akashi, S.; Furuya, M.; Fukuhara, K. I. Rapid confirmation and revision of the primary structure of bovine serum albumin by ESIMS and FRIT-FAB LC/MS. *Biochem. Biophys. Res. Commun.* **1990**, *173*, 639−646.

(30) Cooper, A.; McAuley-Hecht, K. E. Microcalorimetry and the molecular recognition of peptides and proteins. *Philos. Trans. R. Soc. London A* **1993**, *345*, 23−35.

(31) Kevelam, J.; van Breemen, J. F. L.; Blokzijl, W.; Engberts, J. B. F. N. Polymer-surfactant interactions studied by titration microcalorimetry: influence of polymer hydrophobicity, electrostatic forces, and surfactant aggregational state. *Langmuir* **1996**, *12*, 4709−4717.

(32) Nielsen, A. D.; Borch, K.; Westh, P. Thermochemistry of the specific binding of C12 surfactants to bovine serum albumin. *Biochim. Biophys. Acta* **2000**, *1479*, 321−331.

(33) McManus, J. P.; Davis, K. G.; Lilley, T. H.; Haslam, E. The association of proteins with polyphenols. *J. Chem. Soc., Chem. Commun.* **1981**, 309−311.

(34) Kawamoto, H.; Mizutani, K.; Nakatsubo, F. Binding nature and denaturation of protein during interaction with galloylglucose. *Phytochemistry* **1997**, *46*, 473−478.
(35) Self, R.; Eagles, J. Galletti, G. C.; Mueller-Harvey, I.; Hartley, R. D.; Lea, A. G. H.; Magnolato, D.; Richli, U.; Gujer, R.; Haslam, E. Fast atom bombardment mass spectrometry of polyphenols (*syn.* vegetable tannins). *Biomed. Environ. Mass Spectrom.* **1986**, *13*, 449−468.
(36) Jäderlund, O.; Plant, J. da Silva Filho, J. C.; Ferreira, P.; Hayes, W.; Evason, D. J.; Mueller-Harvey, I. MALDI-ToF MS analysis of condensed and hydrolyzable tannins. *Polyphenols Commun.* **2002**, *2*, 491−492.
(37) Vivas, N.; Bourgeois, G.; Vitry, C.; Glories, Y.; de Freitas, V. Determination of the composition of commercial tannin extracts by liquid secondary ion mass spectrometry (LSIMS). *J. Sci. Food Agric.* **1996**, *72*, 309−317.

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