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Interactions of tea tannins and condensed tannins with proteins

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ABSTRACT

Binding parameters for the interactions of four types of tannins: tea catechins, grape seed proanthocyanidins, mimosa 5-deoxy proanthocyanidins, and sorghum procyanidins (mDP = 17), with gelatin and bovine serum albumin (BSA) have been determined from isothermal titration calorimetry data. Equilibrium binding constants determined for the interaction with gelatin were in the range 10^4 to 10^6 M^{-1} and in the order: sorghum procyanidins > grape seed proanthocyanidins > mimosa 5-deoxy proanthocyanidins > tea catechins. Interaction with BSA was generally weaker, with equilibrium binding constants of $\leq 10^3$ M^{-1} for grape seed proanthocyanidins, mimosa 5-deoxy proanthocyanidins and tea catechins, and 10^4 M^{-1} for the sorghum procyanidins. In all cases the interactions with proteins were exothermic and involved multiple binding sites on the protein. The data are discussed in relation to the structures and the known nutritional effects of the condensed tannins.

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1. Introduction

Tannins are a diverse group of polyphenols that are formed as secondary metabolites in plants [1,2] and include a wide range of oligomeric and polymeric polyphenols. Condensed tannins (*syn.* proanthocyanidins), gallotannins and ellagitannins are the most widely occurring tannins. In a previous paper we described the binding of hydrolysable tannins (i.e. gallotannins and ellagitannins) to proteins by isothermal titration calorimetry (ITC), and were able to relate binding parameters to the structural flexibility of the tannin molecules [3]. Here we describe studies of the interactions of flavanol gallates and condensed tannins with proteins.

Condensed tannins are oligomers or polymers of flavonoid units. They occur in many fruits and drinks, such as tea, beer, wine and juices, making them significant in human nutrition [4,5], but are also widely distributed in various browse plants and a few fodder legumes where they can make an important contribution to animal nutrition and health [6]. Condensed tannins are considered to inhibit the digestion of protein and fibre in humans and non-ruminants, acting within the digestive tract to bind to dietary protein and to digestive enzymes [7]. In contrast to this antinutritional effect, condensed tannins have been demonstrated to exhibit numerous biological and pharmacological activities that are of interest in human and veterinary medicine, such as inhibition of lipid oxidation, mutagenicity of carcinogens and tumor promotion [8–12]. Indeed, the observed health benefits of tea consumption and the explanation for the French paradox have been both associated with the beneficial bioactivities of tannins [13].

It is likely that, alongside their well documented antioxidant properties, the interaction of tannins with proteins is fundamental to their observed biological activities [14]. Therefore, a better understanding of this interaction will enable clearer explanations for the biological and pharmacological activities of tannins. In previous work we have utilized ITC for the investigation of tannin–protein interactions involving several hydrolysable tannins [3,15] and epicatechin [16]. In the present study, ITC has been employed to characterize the binding of selected condensed tannins to gelatin and bovine serum albumin (BSA). Gelatin is proline-rich, has an open random coil conformation and is a model for seed prolamins and salivary proline-rich proteins, while BSA is a well characterized model globular protein. Both gelatin and BSA have been commonly used in the literature for investigation of relative binding affinities of tannins.

Condensed tannins from four sources have been studied here; Fig. 1 depicts examples of some of their typical structures. Green tea catechins are principally composed of epigallocatechin-3-gallate

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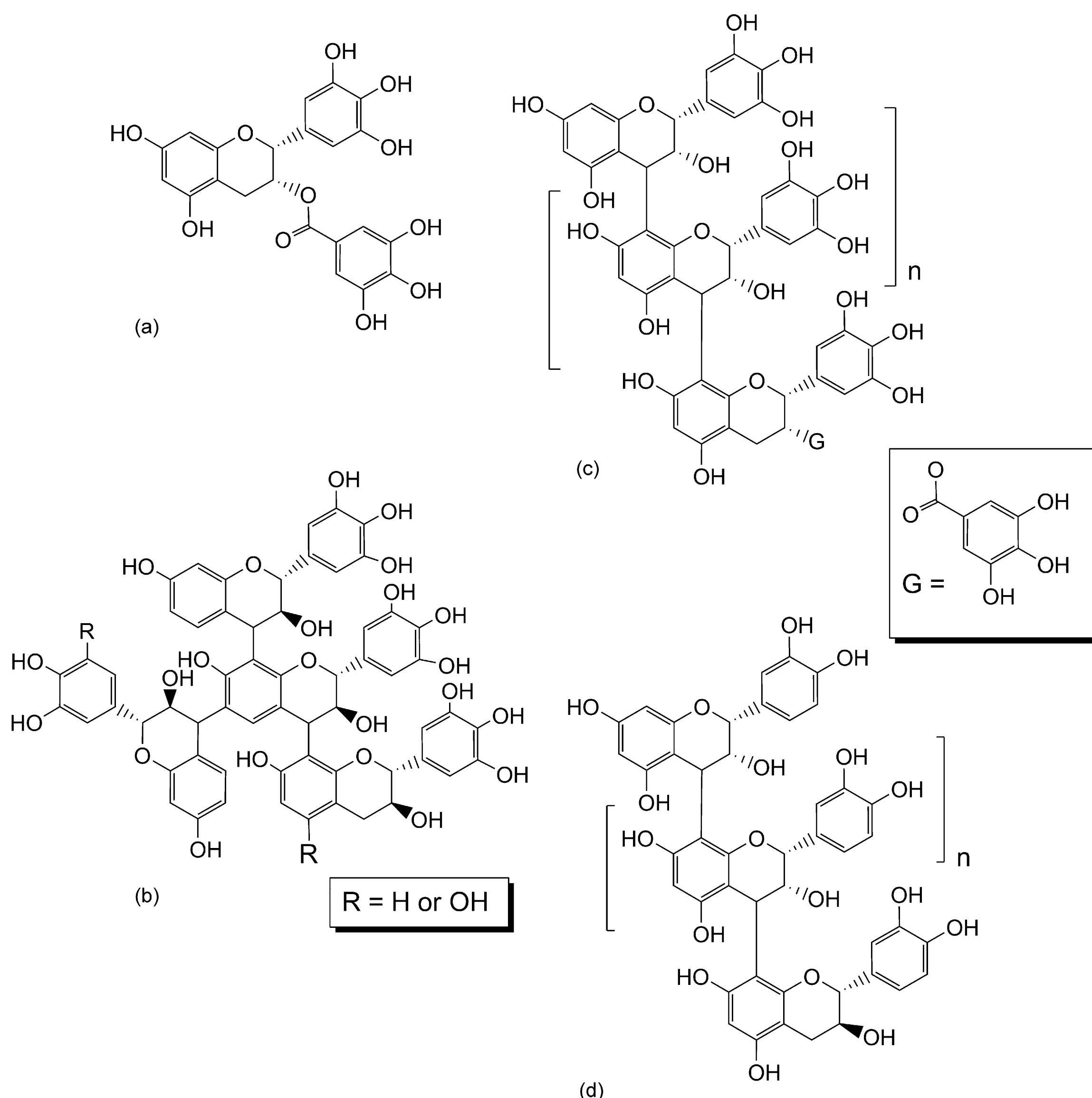

Fig. 1. Typical chemical structures of the tannins studied: (a) flavanol gallate from tea, (b) profisetinidins (R = H) and prorobinetinidins (R = OH) from mimosa bark, (c) galloylated prodelphinidin from grape seeds, and (d) procyanidins from sorghum. Structures (a)–(d) are well described in the literature and were confirmed by MALDI-TOF MS of the isolated tannin mixtures.

(EGCG), as well as epicatechin-3-gallate, epigallocatechin, epicatechin and catechin, and are widely reported for their health benefits in the human diet [8,10,12]. Grape seed tannins consist of oligomeric and polymeric procyanidins, which can be galloylated to various degrees [17–19]. Like the tea catechins, they are perceived as beneficial to health [20,21]. Mimosa tannins are predominantly oligomers or polymers of fisetinidol and robinetinidol units. Fisetinidol and robinetinidol units differ from grape seed and sorghum flavanol units by not having a phenolic group at the C-5 position of the A-ring. No information is available on the nutritional value of mimosa tannins. However, sorghum tannins have been observed to lower protein digestibility [22].

2. Materials and methods

2.1. Materials

BSA (purity ≥99%, essentially globulin free, 66 kDa) and bovine skin gelatin (100 kDa) were purchased from Sigma (Poole, Dorset, U.K.). The sorghum procyanidin sample (mDP = 17) (M_r 4930) was donated by Professor Ann Hagerman and was purified from *Sorghum bicolor* grain [23]. Tea catechins were isolated from TEAMAX-AR25 and grape seed proanthocyanidins from GRAPEMAX EXTRA PURE (Burgundy Botanical Extracts, Reyssouze, France). 5-Deoxy proanthocyanidins were isolated from mimosa tannins (*Acacia mearnsii*), which were donated by Forestal Quebracho Ltd. All solutions for ITC analysis were prepared in 50 mM citrate buffer at pH 6 and were degassed under vacuum prior to use.

2.2. Isolation of tannins

Tannins were isolated from commercially available tannin products by chromatography on Sephadex LH-20 (Amersham Biosciences, Chalfont St. Giles, Buckinghamshire, U.K.) as has been previously described [3]. 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 pre-swollen in 10 cm^3 methanol/water, 1:1, v/v; column dimensions: 10 cm length × 1.5 cm diameter). Non-tannin

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.

2.3. Molecular weight characterization of tannins by gel permeation chromatography

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, U.K.). 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). 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.

2.4. MALDI-TOF MS analysis of grape seed tannins

MALDI-TOF mass spectra were collected on a Bruker Daltonics Reflex III-TOF mass spectrometer (Bruker Daltonics, Coventry, U.K.) equipped with delay extraction and a N_2 laser set at 337 nm. In the positive reflectron mode an accelerating voltage of 25.0 kV and a reflectron voltage of 26.3 kV were used. All spectra were the sum of 200 shots. Spectra were calibrated with ClinProt Standards, containing protein calibration standards and peptide calibration standards (Bruker).

The matrix known as super-DHB was prepared by mixing 2,5-dihydroxybenzoic acid (2,5-DHB) with 2-dihydroxy-5-methoxybenzoic acid (9:1, w:w) in acetonitrile: 0.1% TFA in water (7:3, v/v) (20 mg cm^{-3}). The dried tannin sample was reconstituted in acetonitrile (4 mg cm^{-3}) and the matrix mixed with 0.2 M NaCl (1:1, v/v). The sample solution was then mixed with the matrix solution (1:1, v/v) and 0.8 μl of the final mixture was applied on the target and left to dry (dried droplet method).

2.5. MALDI-TOF MS of mimosa tannins

Tannins (18 mg cm^{-3}) were first dissolved in acetone/water (4:1; v/v); the solvent also contained NaCl (18 mg cm^{-3}). Then five parts of a trans-3-indole acrylic acid solution (75 mg in 1 cm^3 tetrahydrofuran) was mixed with 1 part of the tannin solution. The sample platten was loaded with 0.4 μl of the final sample mixture and the tannin samples were analysed using an SAI LT3 Laser TOF mass spectrometer (Scientific Analysis Instruments Ltd, Manchester, U.K.) equipped with a nitrogen laser operating at 337 nm. Spectra were recorded in linear mode employing delayed extraction with a delay time of 75 ns, resulting in a mass focusing at 1000 amu. Each spectrum was obtained by allowing the laser pulses to scan the whole sample spot in a straight line, moving through a total of 60 discrete positions and firing 8 shots at every position.

2.6. Isothermal titration calorimetry

A TA Instruments Nano ITC instrument (TA Instruments Ltd., Crawley, West Sussex, U.K.) was used to measure enthalpy changes associated with tannin–protein interactions at 298 K. In a typical experiment, buffered gelatin or BSA solution 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 [24]. 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 [15]. 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 [15]. 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.

2.7. 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, U.S.A.). Data fits were obtained using the independent set of multiple binding sites model, for which the analytical solution for the total heat measured (Q) is determined by the formula:

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

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 [25]. 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 ($p < 0.05$). Free energy, ΔG, was determined from the binding constant ($\Delta G = -RT \ln K$, where R is the gas constant and T is the absolute temperature in Kelvin) and entropy, ΔS, from the second law of thermodynamics ($\Delta G = \Delta H - T\Delta S$).

3. Results and discussion

3.1. Characterisation of tannin molecular weights

The analysis of tannin molecular weights is not straight forward. As discussed by Taylor et al. [26], either GPC or mass spectrometry can be utilized to determine size distributions within a tannin

Table 1
MALDI-TOF MS peak assignments.

	Major m/z values observed	Calculated m/z values	Assignments
Mimosa tannins:			
3-mers:	890.0, 906.1	889.83; 905.83	3R; 2R + 1EG
4-mers:	1178.7, 1194.7	1178.11; 1194.44	4R; 3R + 1EG
5-mers:	1467.1, 1483.1	1466.39; 1482.39	5R; 4R + 1EG
Grape seed tannins:			
2-mers + g:	753.1	753.55	2ECg
3-mers:	889.2, 905.1	889.83, 905.83	3EC; 2EC + 1EG
3-mers + g:	1041.2	1041.83	3ECg
4-mers:	1177.3, 1193.2	1178.11, 1194.11	4EC; 3EC + 1EG
4-mers + g:	1329.3	1330.11	4ECg
5-mers:	1465.3, 1481.3	1466.39, 1482.39	5EC; 4EC + 1GC
5-mers + g:	1617.4	1618.39	5ECg

R = robinetinidol; EG = gallocatechin and/or epigallocatechin; EC = catechin and/or epicatechin; g = galloyl.

mixture. However, mass spectra can be dominated by lower molecular weight species, despite sometimes contrary evidence from chemical methods indicating an abundance of larger molecules; this is observed for the analysis of tannins from several sources by different MS methods [27]. GPC results, on the other hand, are influenced not only by molecular size, but also by molecular shape and hydrodynamic radius, which both can differ between tannins and the polystyrene standards. Therefore, neither approach is completely without error. For consistency between samples, it was decided here to use the peak molecular weights (M_p) from GPC data in ITC data analysis.

MALDI-TOF MS data agreed with the M_p estimates for mimosa and grape seed tannins, and the peak assignments for these tannins are given in Table 1. The main MALDI-TOF MS peaks of mimosa tannins could be assigned to trimers, tetramers and pentamers of prorobinetinidins, and peaks of grape seed tannins to procyanidins and galloylated procyanidins ranging from dimers to pentamers. MS peaks of galloylated procyanidins were more prominent than non-galloylated peaks in the MALDI-TOF spectra.

The sorghum tannin was reported to have a molecular weight of 4930 g mol^{-1} and mean degree of polymerization (mDP) of 17 by Hagerman et al. [23]; GPC analysis confirmed both the molecular weight and mDP. The sorghum tannin polymer contains 16 epicatechin units and a terminal catechin unit. The tea tannin M_p value (710 g mol^{-1}) was an overestimate in comparison to the calculated 458 g mol^{-1}, which could reflect the presence of oxidized material or the influence of molecular shape and hydrodynamic radius on the GPC data.

3.2. Interaction with gelatin

Fig. 2 shows the ITC binding isotherms for the interaction of the condensed tannins with gelatin as plots of observed change in enthalpy (ΔH_{obs}) versus tannin:protein molar ratio. Each plot shows an exothermic interaction in which the protein binding sites become completely saturated at high tannin:protein molar ratios. The high molar ratio values required for saturation suggest multiple binding sites of tannin to protein, which is confirmed by the n-values obtained when the data were fitted to a binding model comprised of an independent set of multiple binding sites. As summarized in Table 2, binding stoichiometries (n, tannin:protein) were in the range of 16:1–71:1 and revealed a non-linear trend of decreasing n with increasing molecular weight.

The equilibrium binding constants (K) for the interactions of each tannin with gelatin are also summarized in Table 2, and ranged from 9.8×10^3 M^{-1} to 2.0×10^6 M^{-1} in the order tea catechins < mimosa < grape seed < sorghum tannins. The highest n-value and lowest binding constant belonged to the tea catechins. The major tea tannin is EGCG (see Fig. 1); in a previous study it was shown that EGCG binds to proline-rich β-casein in a multidentate fashion to multiple sites on the protein surface, since each proline and aromatic ring offers a potential binding site [28]. Given the high n-values for the other condensed tannins, it is likely that the same is true for these molecules and that the n-value is limited by the size of the tannin molecule since larger molecules can occupy a greater number of binding sites on the protein. It is interesting to note that the condensed tannins with reputed health

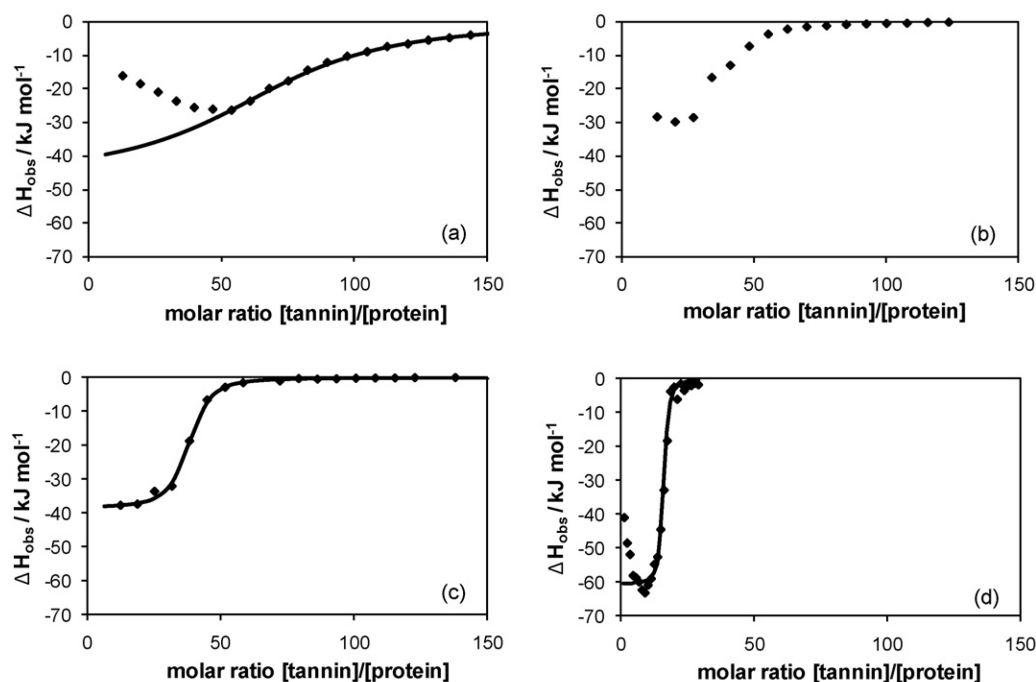

Fig. 2. Typical ITC binding isotherms for (a) tea tannins, (b) mimosa 5-deoxy proanthocyanidins, (c) grape seed proanthocyanidins and (d) sorghum procyanidins interactions with gelatin. Experimental data are depicted as points and the binding models are depicted as solid lines. Binding model parameters are summarized in Table 2.

Table 2
Estimated thermodynamic binding parameters for the interaction of condensed tannins with gelatin and BSA.

	Tea catechins	Mimosa 5-deoxy proanthocyanidins	Grape seed proanthocyanidins	Sorghum procyanidins
M_p/g mol^{-1}	710	1126	1206	4930[a]
Gelatin:				
n	71	34	35	16
K/M^{-1}	9.8×10^3	8.6×10^4	3.3×10^5	2.0×10^6
ΔH/kJ mol^{-1}	−45.5	−35.4	−38.0	−60.3
ΔG/kJ mol^{-1}	−22.7	−28.2	−31.5	−35.9
ΔS/J mol^{-1} K^{-1}	−76.5	−24.2	−21.8	−81.9
BSA:				
n	18.5	39	7	11
K/M^{-1}	136	5.9×10^3	1.5×10^3	3.3×10^4
ΔH/kJ mol^{-1}	−154	−9.8	−102	−21.4
ΔG/kJ mol^{-1}	−12.2	−21.5	−18.1	−27.1
ΔS/J mol^{-1} K^{-1}	−476	39.3	−282	19.1

[a] M_r value reported by Hagerman et al. [23].

benefits (tea catechins, grape seed proanthocyanidins) possessed equilibrium binding constants at least one order of magnitude lower than sorghum tannins, which are perceived as antinutritional with respect to protein digestibility. As well as having less negative effects on protein digestibility, weaker interactions may allow improved uptake of the beneficial condensed tannins *in vivo*.

The derived parameters of free energy (ΔG) and entropy (ΔS) are also presented in Table 2. Free energy was similar at each concentration and was negative, which is a requirement for a spontaneous biomolecular interaction [24]. In each case the entropy term (ΔS) was also negative, indicating an increase in molecular order due to binding, which would occur upon aggregation and may also imply a role for hydrogen bonding in the formation of the complex [16]. Indeed, a study by Simon et al. [29] on the binding of wine tannins to a proline rich salivary protein fragment concluded that hydrogen bonding between proline carbonyls and phenol or catechol OH groups was the principal driving force for interaction. Butler et al. [22] also concluded that hydrogen bonding was predominant in binding of sorghum tannins to proline-rich proteins. A dominant role of hydrogen bonding could account for the two-orders of magnitude difference in binding constants between sorghum tannins and mimosa tannins, where a key structural difference is the lack of one phenolic group at C-5 in the latter's monomer unit. Therefore, it would be expected that sorghum tannins would have stronger binding based on hydrogen bonding simply based on the number of available groups to participate in hydrogen bonding. Differences in structural flexibility may also be a contributing factor to the difference in binding constants between sorghum tannins and mimosa tannins since 5-deoxy proanthocyanidins are likely to be more flexible than the procyanidins around the C–C bonds between the monomers, again by virtue of the missing phenolic group at the C-5 position.

The difference in binding constants between the tea catechins and grape seed proanthocyanidins was also two-orders of magnitude and may be due to a molecular weight effect. Sarni-Manchado et al. [30] reported that protein binding of polymeric condensed tannins was stronger than that of low molecular weight oligomers and monomers. Indeed, it is a widely held assumption that higher molecular weight tannins, because they precipitate proteins more readily than monomeric flavanols, are able to bind more strongly

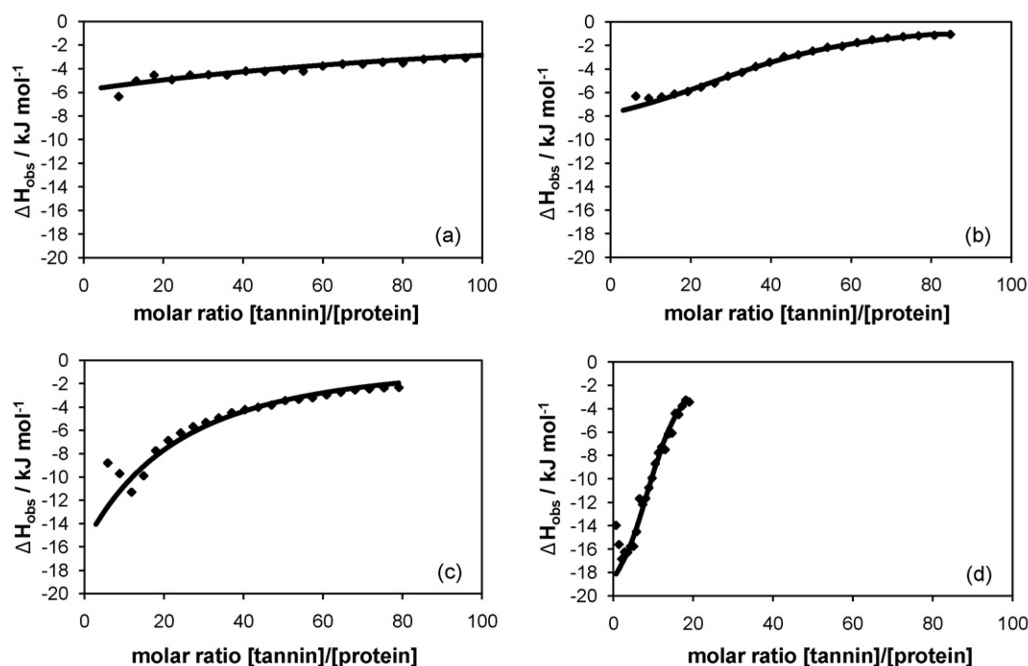

Fig. 3. Typical ITC binding isotherms for (a) tea tannins, (b) mimosa 5-deoxy proanthocyanidins, (c) grape seed proanthocyanidins and (d) sorghum procyanidins interactions with BSA. Experimental data are depicted as points and the binding models are depicted as solid lines. Binding model parameters are summarized in Table 2.

or preferentially to proteins [14], and this assumption appears valid for the binding to gelatin of the selected condensed tannins studied here.

3.3. Interaction with BSA

The ITC data for the interaction of the condensed tannins with BSA are shown in Fig. 3 and analysis of these data shown in Table 2 revealed very weak interactions in all cases ($\leq 10^3$ M^{-1}), apart from sorghum tannins that gave an equilibrium binding constant of 3.3×10^4 M^{-1}. Nevertheless, these data are consistent with the early work of Asquith and Butler [31] who found no significant difference in the relative binding affinities of a range of condensed tannins with BSA. As for their interaction with gelatin, the flavanol gallates (tea and grape seed tannins) appeared to interact mostly by hydrogen bonding to BSA. However, the interactions of both sorghum and mimosa tannins with BSA were found to be entropically driven (i.e. positive ΔS values), in contrast to their enthalpically driven interactions with gelatin. The dominance of entropy in the interaction suggests that hydrophobic interactions were dominant in the formation of complexes [14]. This finding conflicts with previous experimental evidence that suggested hydrogen-bonding was predominant for sorghum tannin binding to BSA [23], although it must be noted that those data took into account the effects of varying binding conditions, including the influence of solvents and temperature, that are not considered here. It is therefore most likely that binding involves a balance of hydrophobic interactions and hydrogen bonding as has been suggested in earlier studies [14,22].

3.4. Comparison of condensed tannin binding to BSA and gelatin

The data presented here show that binding constants of condensed tannins with gelatin are by one to two orders of magnitude greater compared to BSA. This agrees with previous studies that have shown preferential interaction of condensed tannins with gelatin in competition with BSA [31]. Hydrogen bonding is clearly a dominant factor in the binding of condensed tannins to gelatin, whereas it is less clear whether hydrogen bonding or hydrophobic interactions predominate for the weaker binding to BSA. Molecular weight effects also appear to be important for the binding of condensed tannins to proteins, whereas in our previous work molecular weight was not a dominant factor determining the strength of hydrolysable tannin binding to proteins [3].

In our earlier studies of hydrolysable tannins binding to gelatin and BSA, it was noted that structurally flexible gallotannins showed less difference in their relative binding strengths to gelatin and BSA, whereas the less flexible ellagitannins exhibited stronger binding to gelatin than to BSA [3]. Therefore, a lack of structural flexibility in the condensed tannins may be a contributing factor to their low affinity for BSA.

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