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# Interactions between Flavan-3-ols and Poly(L-proline) Studied by Isothermal Titration Calorimetry: Effect of the Tannin Structure

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Interactions of proline-rich proteins (PRPs) with flavan-3-ols was studied using poly(L-proline) as a model protein by means of isothermal titration calorimetry (ITC). Several parameters were varied: (i) the galloylation and B-ring trihydroxylation of the flavan-3-ols (catechin, epicatechin, epicatechin gallate, and epigallocatechin gallate) and (ii) the degree of polymerization (monomers were compared to a mixture of oligomers with average degree of polymerization of 3.85). Large differences were observed between the flavan-3-ol monomers: no enthalpy change was measured when catechin and epicatechin were titrated by poly(L-proline), whereas thermodynamic parameters were determined in the case of galloylated monomers and mixture of oligomers. Stoichiometry ranged from 1 oligomer bound for each 12 proline units to 1 galloylated monomer for each 8 or 10 proline units. Association constants were in the range of $10^4$–$10^5$ $M^{-1}$, indicating a relatively high affinity of galloylated flavanols toward poly(L-proline), and the coexistence of both enthalpy- and entropy-driven phenomena was suggested. Finally, the binding of grape seed tannins to proteins was shown to be a cooperative process.

**KEYWORDS: Tannins; proline-rich proteins; titration microcalorimetry; interactions**

## INTRODUCTION

Polyphenols are plant compounds present in foods and beverages. Among them, flavan-3-ols are particularly abundant in tea and are constituent units of condensed tannins (proanthocyanidins) found in most fruits and in wines or cocoa (**Figure 1**; **Table 1**). Interest in proanthocyanidins is related to their quantitative and qualitative importance for technological and organoleptic properties, especially in beverages such as wine, cider, and beer. It is well-known that tannins interact with proteins (*1*–*6*). This characteristic is involved in astringency (*7*, *8*) and exploited in protein fining treatments (*8*).

In an attempt to study the ability of proteins and polyphenols to form soluble complexes and colloidal aggregates, biomimetic models were developed in a previous study (*9*). They included, on the one hand, a series of flavan-3-ol monomers differing by structural features expected to influence their affinity for proteins and, on the other hand, a poly(L-proline) having a composition reminiscent of Pro-Pro sequences found in salivary proline-rich proteins (PRP) and in gelatin used in wine fining. Their ability to interact and form colloidal aggregates, as well as the stability of the systems, was investigated by means of dynamic light scattering (DLS) and UV–visible spectroscopy, whereas the morphology and size of the aggregates were studied by cryotransmission electron microscopy (cryo-TEM). Strong differences were observed between epicatechin (Ec) and epigallocatechin (Egc), which did not form aggregates with poly(L-proline), and epicatechin gallate (EcG), epigallocatechin gallate (EgcG), and catechin (Cat), which did. This study highlighted the strong influence of structural details on the interactions. When complexes were formed, their stability depended on the monomer/protein ratio and on the initial protein concentration.

Several techniques have already been used to study the interactions between polyphenols and proteins: NMR (*10*–*12*), circular dichroism (*13*), mass spectrometry (*14*), and UV–visible and fluorescence spectroscopy (*15, 16*). The main features of polyphenol structure and properties that influence interactions between tannins and proline-rich proteins have been reported to be as follows:

(i) Average degree of polymerization (DP): increasing the mean molecular mass of grape procyanidins increases their ability to precipitate gelatin and salivary proteins (*8, 17, 18*), as well as their perceived astringency (*19*), up to a given degree of polymerization.

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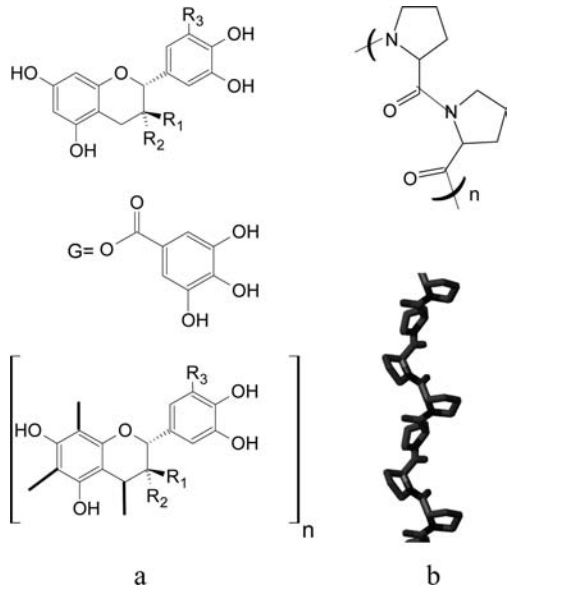


**Figure 1.** Structures of (**a**) the monomers and grape seed tannins ($n$ |= 3.85). Bold bonds in the condensed tannin structure correspond to the interflavanal linkages, between carbons C4 and C6 or C4 and C8. **Table 1** gives the monomer names when $R_1$, $R_2$, and $R_3$ vary. (**b**) Poly(L-proline). PPII helix is shown.

**Table 1.** Molecular Formulas and Abbreviations of the Studied Polyphenols

| flavan-3-ol | $R_1$ | $R_2$ | $R_3$ | mol mass (Da) | partition coefficient (octan-1-ol/ water) |
|---|---|---|---|---|---|
| catechin (Cat) | OH | H | H | 290 | 2.37 |
| epicatechin (Ec) | H | OH | H | 290 | 1.34 |
| epigallocatechin (Egc) | H | OH | OH | 306 | 0.15 |
| epigallocatechin gallate (EgcG) | H | G | OH | 458 | 9.1 |
| epicatechin gallate (EcG) | H | G | H | 442 | 26.5 |

[a] $R_1$, $R_2$, and $R_3$ are the same as in **Figure 1**.

(ii) Galloylation: increasing the number of galloyl ester groups on the galloyl-D-glucose series leads to an increase in protein-binding capacity (*4*), and galloylation of flavan-3-ol induces the formation of complexes with proline-rich proteins more easily (*9, 14*). Astringency is consequently also influenced by galloylation (*19*).

In parallel, amino acids involved in polyphenol-PRP interactions have been shown to belong to Pro-Pro sequences (*20*), even though Simon et al. (*21*) also highlighted the role played by adjacent glycine residues. In the present study, a synthetic poly(L-proline) was used as a model for PRP. Isothermal titration calorimetry (ITC) was used to characterize the protein binding of four different flavan-3-ol monomers (Cat, Ec, EcG, EgcG), and a oligomeric fraction of grape seed tannins on poly(L-proline). ITC is a powerful technique that allows the study of the thermodynamics of protein–ligand interactions (*22–24*) because most reversible biomolecular interactions involve changes in enthalpy (gain or loss of heat energy). Furthermore, this technique allows determination of the binding constant and stoichiometry of an interaction in solution without chemical modification or immobilization of either species. Our objectives in this study were to rank the monomers and other flavanol derivatives according to their affinity toward proline-rich proteins, to establish a stoichiometry for interactions between flavan-3-ols and poly(L-proline), and, because tannin-protein interactions are reported to involve both hydrophobic effects and hydrogen bonding, to determine tannins' structural key parameters for protein binding.

In this study, experiments were performed at an acidic pH of 3.6, which is a relevant pH for wine. We checked that, when sipping wine, the pH of the expectorated solution is equal to that of wine and not to that of saliva (neutral pH).

## MATERIALS AND METHODS

**Chemicals.** Deionized water was obtained with a Milli-Q system (Millipore, Billerica, MA). Catechin, epicatechin, epicatechin gallate, and epigallocatechin gallate (**Table 1**) were purchased from Sigma-Aldrich (St. Louis, MO), checked by HPLC, and used without any further purification. Poly(L-proline) (molecular mass, 6900 Da; $DP_n$, 71) was obtained from Sigma-Aldrich. Ammonium acetate was purchased from Merck (Whitehouse Station, NJ). The tannin fraction was prepared from grape seeds (*Vitis vinifera* var. Shiraz) as described before (*25*). Its average degree of polymerization was determined using HPLC analysis after thiolysis. This method is based on acid-catalyzed cleavage of the interflavanol linkages in the presence of a nucleophilic agent (toluene-α-thiol), followed by HPLC analysis of the reaction products. It gives access to the nature and proportions of the different constitutive units, thus allowing the calculation of the fraction average degree of polymerization. However, this method does not provide molecular weight distribution of the tannin fraction. The fraction used here was shown to contain 14% of epicatechin gallate units and to present an average degree of polymerization of 3.8 and is referred to as DP4.

**Titration Microcalorimetry.** A VP-ITC instrument (MicroCal, Northampton, MA) was used to measure enthalpy changes associated with protein-tannin interactions at 298 K. Poly(L-proline) and polyphenols were dissolved in the same buffer, that is, 10 mM ammonium acetate at pH 3.6 (pH adjusted with acetic acid). All solutions were degassed prior to the measurements. In a typical experiment, poly(L-proline) solution (0.028–0.056 mM) is placed in the 1.448 mL sample cell of the calorimeter and tannin solution (typically 4 mM) is loaded into the injection syringe. Tannin solution is titrated into the sample cell as a sequence of 30 injections of 10 $\mu$L aliquots. The duration of each injection is 20 s, and the time delay (to allow equilibration) between successive injections is 5 min The contents of the sample cell are stirred throughout the experiment at 300 rpm to ensure thorough mixing.

Raw data obtained as a plot of heat flow (microcalories per second) against time (minutes) are then integrated peak-by-peak and normalized to obtain a plot of observed enthalpy change per mole of injectant ($\Delta H$, kcal $mol^{-1}$) against the molar ratio (tannin/protein). Peak integration is performed using Microcal Origin (Microcal Software, Northampton, MA). Control experiments include the titration of monomers and proanthocyanidin fraction into buffer and are subtracted from titration experiments. The experimental data are fitted to a theoretical titration curve using Microcal Origin, with $\Delta H$ (enthalpy change), $K_a$ (association constant), and $n$ (number of binding sites per molecule) as adjustable parameters, from the relationship

$$Q_i = \frac{nP_t\Delta HV_0}{2}\left[1+\frac{A_t}{nP_t}+\frac{1}{nK_aP_t}-\sqrt{\left(1+\frac{A_t}{nP_t}+\frac{1}{nK_aP_t}\right)^2-4\frac{A_t}{nP_t}}\right] \quad (1)$$

where $P_t$ is the total protein concentration, $A_t$ is the total concentration of the ligand, $V_0$ is the volume of the cell, and $Q_i$ is the total heat released for injection $i$. $\Delta G$ values and entropy contributions can be then determined from the standard equation

$$\Delta G = -RT\ln K_a = \Delta H - T\Delta S \quad (2)$$

where $\Delta G$, $\Delta H$, and $\Delta S$ are the changes in, respectively, Gibbs free energy, enthalpy, and entropy of binding, $T$ is the absolute temperature, $R$ |m= 8.32 J $mol^{-1}$ $K^{-1}$, and $K_a$ is the association constant.

## RESULTS AND DISCUSSION

Although ITC is a powerful technique to study the thermodynamics of protein–ligand interactions, calorimetric isotherms

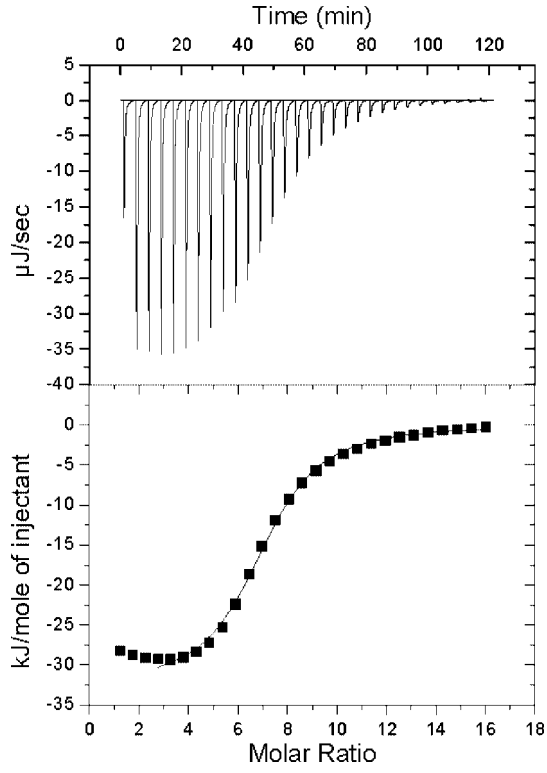


**Figure 2.** (Top) Raw data plot of heat flow against time for the titration of 4 mM epicatechin gallate into 0.056 mM poly(L-proline). (Bottom) Corresponding plot after integration of peak areas and normalization to yield a plot of molar enthalpy change against flavan-3-ol/poly(L-proline) ratio. The one-site fit curve is displayed as a thin line. Experiments were done in triplicate.

must be interpreted carefully, because changes in enthalpy are likely to arise from a combination of several phenomena: binding of ligands to proteins, aggregation of the complexes thus formed (*26*), and also conformation changes of the ligand and/or the protein (*27*), as well as ligand and/or protein self-association/dissociation. Moreover, there are some limitations when low-affinity systems are studied, which can be overcome, provided the complex stoichiometry is well characterized, as shown by Turnbull et al. (*28*). An attempt to titrate recombinant human salivary proline-rich proteins was done by Pascal et al. (*27*), but unfortunately the data were difficult to interpret, since human PRPs may undergo conformational changes such as disorder to order transitions upon ligand addition.

**Galloylated/Nongalloylated Monomers.** In a previous paper, it was shown that nongalloylated monomers (Ec and Egc) have a totally different behavior compared to EcG and EgcG when they are mixed with a poly(L-proline) solution (*9*): Ec and Egc did not induce particle formation, whereas EcG and EgcG did. In the case of catechin, particles were formed after a lag phase, but at polyphenol/protein ratios much higher than with galloylated monomers. Mechanisms involved in the particle formation may thus be different. To check this hypothesis, isothermal titration calorimetry experiments were performed.

The titration of 0.056 mM poly(L-proline) by EcG 4 mM is shown in **Figure 2**: the curve is typical of enthalpy-driven protein–ligand interactions, with relatively sharp decreasing exothermic peaks upon flavan-3-ol addition. As the ligand concentration increases, the number of available binding sites on the poly(L-proline) decreases; hence, the exothermic contribution to the enthalpy changes associated with binding is decreased. Eventually, all of the binding sites on poly(L-proline) are saturated, and addition of more flavan-3-ol leads to a plateau;

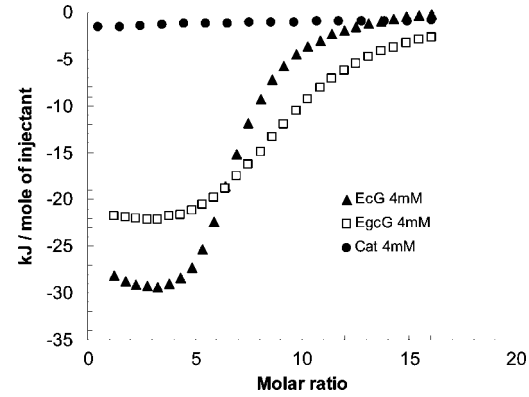


**Figure 3.** Titration plots of catechin (●), epicatechin gallate (▲), and epigallocatechin gallate (□) into poly(L-proline). $\Delta H$ is plotted against the flavan-3-ol/poly(L-proline) ratio. Experiments were done in duplicate (Cat) or triplicate (EgcG, EcG).

the enthalpy changes measured correspond to the monomer dilution ($\Delta H$ |ad 0 when the blank titration is subtracted.

In **Figure 3** we compare the curves obtained when 4 mM solutions of different flavan-3-ol monomers were titrated into 0.035 mM poly(L-proline) solutions. The heat changes observed when catechin or epicatechin (data not shown) was added are negligible compared to those observed with galloylated monomers. Nongalloylated monomers do not interact with poly(L-proline), which is in agreement with some previous studies conducted on other proline-rich proteins (*20*, *29*). Charlton et al. (*30*) obtained similar results when they studied the binding of polyphenols to proline-rich peptides by NMR with glucose-based esters of gallic acid. They found that the binding capacity increased with the number of aromatic rings (trigalloylglucopyranose < tetragalloylglucopyranose < pentagalloylglucopyranose). This phenomenon was attributed first to an increased hydrophobicity of the molecules with size and galloylation (*20*), along with the formation of multiple bonds.

When one is dealing with nongalloylated monomers, rings A and B are the only sites susceptible to being involved in hydrophobic interactions. Ring D can play an additional role in the case of EcG or EgcG (*11*). Charlton et al. studied the interactions between a model proline-rich heptapeptide Gln-Gly Arg-Pro-Pro-Gln-Gly and EgcG and determined by means of NMR and molecular modeling that both rings A and D have stacking interactions, with Pro5 and Pro4, respectively.

**Dihydroxylation/Trihydroxylation of B Ring.** The raw data (example **Figure 2**) suggest that the phenomena occurring are simpler that what was observed in the case of the human PRP IB-5 (*27*), with relatively sharp exothermic peaks. In some conditions, the first five or six injections of ligand into poly(L-proline) lead to a slight increase of exothermic peaks $\Delta$, before the classical $\Delta$ decrease in heat release.

In our system, poly (L-proline), the conformation of which is described as a helix, is expected to be a rather rigid macromolecule, and is not likely to intramolecularly reorganize or undergo conformational changes upon flavan-3-ol addition. The signal can thus be essentially mainly attributed to ligand binding to the macromolecule. This is one large difference with human proline-rich salivary proteins, which undergo conformational changes. However, the purpose of this study was to quantify and to rank binding affinity of polyphenols toward proline-rich proteins, and by using poly(L-proline) we get a simple signal that allows the determination of binding constants. Furthermore, it is worth noting that preliminary small-angle

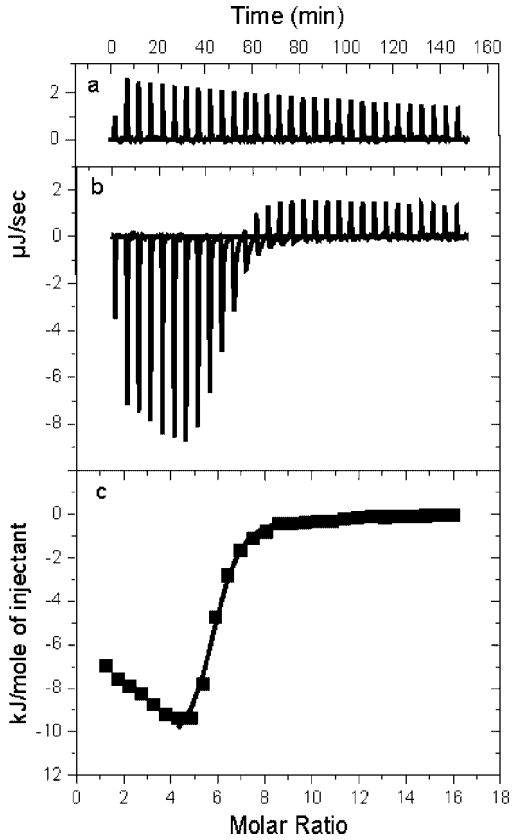


**Figure 4.** (**a**) Blank experiment for injection of DP4 4 mM in buffer. (**b**) Titration plot of DP4 4 mM into 0.056 mM poly(L-proline). The molar ratio is the number of moles of DP4 divided by the number of moles of poly(L-proline). (**c**) Integration of peaks and fitted curve (thin line) for one-site model. Experiments were done in duplicate.

X-ray scattering experiments performed on human proline-rich salivary proteins have shown that these molecules have a rather elongated conformation in solution, like poly(L-proline).

**Monomers/Polymers.** A titration curve of poly(L-proline) 0.056 mM by a 4 mM solution of DP4 is shown in **Figure 4b**. The experimental data are quite different from those observed with EgcG: the peaks resulting from each injection are exothermic up to the 12th injection, but the heat release first dramatically increases before decreasing after the 7th injection. After the 13th injection, the peaks are endothermic, and their value is quite stable. This happens once all poly(L-proline) binding sites are saturated with DP4 and corresponds to the dilution of DP4 after injection as checked by the control experiment (**Figure 4a**).

The enthalpy increase observed in the early stages of titration has also been mentioned by Frazier et al. (*26*) in the case of hydrolyzable tannins representative of gallo- and ellagitannins (myrobolan and tara tannins) added to gelatin, another proline-rich protein. It suggests a cooperative binding of procyanidins to poly(L-proline): the procyanidins already added in the titration cell and bound to poly(L-proline) have a favorable effect on the new binding interactions. Molecules in the DP4 tannin fraction contain on average 8.2 aromatic rings able to interact with proline residues. This average value is calculated from the average degree of polymerization and proportion of galloylated units established by thiolysis, but DP4 is actually a mixture of oligomers, the size distribution of which cannot be easily determined. The longest oligomers have many potential interaction sites and may bind to several proline clusters, not necessarily on the same protein molecule, possibly allowing a bridging between macromolecules and involving easier interactions between polyphenols and PRPs once the first tannins are bound. Furthermore, if the ligand binds to poly(L-proline) by one end, it is thus in close vicinity to the poly(L-proline), and the probabilities for the procyanidin molecule to bind with a second proline residue increase. Cooperative binding mechanisms have also been reported by Simon et al. (*21*) in the case of B3 (a catechin-catechin dimer) and a human salivary protein fragment.

**Thermodynamic Parameters.** From the titration curves, thermodynamic parameters were calculated (**Table 2**). We present here the results obtained with the one-site model. The fitting process was performed by omitting the first five or six peaks, because in the case of DP4, the first part of the titration presents some cooperative effect. The resulting curve displays good fit with experimental data for the second part of the titration (**Figures 2** and **4c**). The use of a two-site model would allow the whole titration to be fit but would be irrelevant from a biochemical point of view because there are not enough experimental data for fitting six parameters. Constants of association, as well as $\Delta H$, $\Delta S$, and $\Delta G$, could be derived from the one-site model, although the value of $\Delta H$ may not be completely correct due to the exclusion of the first few peaks.

The binding constants followed the order $K_{a,EgcG} < K_{a,EcG} < K_{a,DP4}$. The calculated association constant for the procyanidin oligomer is roughly 1 order of magnitude larger than that calculated for EgcG, 4 times larger than in the case of EcG, which is consistent with what was observed previously: up to a given DP, the tannin affinity for proline-rich proteins increases with its DP (*8*, *17*, *18*). EcG has a higher $K_a$ than EgcG (about double), which is not in agreement with a previous study (*29*). However, this study was done with a pool of salivary proteins and not with poly(L-proline). Salivary proteins contain also glycine residues, which are involved in salivary protein/tannin interaction and may account for the differences observed (*21*). In our previous paper (*9*), we determined partition coefficients of flavan-3-ols between octanol and water and found the same ranking for EgcG and EcG (**Table 1**): Egc < Ec < Cat < EgcG < EcG.

This ranking is consistent with the monomer affinity to proteins and confirms that the less water-soluble ones, which are the most hydrophobic, are the more susceptible to interaction with proteins.

From a thermodynamic point of view, the change in Gibbs free energy results from the sum of an entropic and an enthalpic term. Entropic contributions are usually correlated to hydrophobic interactions, loss of water molecules, loss of ions, and conformational changes, whereas enthalpic contributions are

**Table 2.** Thermodynamic Parameters for the Interaction of EgcG, EcG, and DP4 with Poly(L-proline)[a]

| | $n$ | $K_a$ ($10^4$ $M^{-1}$) | $K_d$ ($10^{-6}$ M) | $\Delta G$ (kJ $mol^{-1}$) | $\Delta H$ (kJ $mol^{-1}$) | $-T\Delta S$ (kJ $mol^{-1}$) |
|---|---|---|---|---|---|---|
| EgcG | $9.1 \pm 0.3$ | $3.7 \pm 0.1$ | $27.3 \pm 0.7$ | $-26 \pm 0.1$ | $\mathbf{-25.1 \pm 0.5}$ | $-0.9 \pm 0.5$ |
| EcG | $6.9 \pm 0.1$ | $8.1 \pm 0.2$ | $12.3 \pm 0.3$ | $-28 \pm 0.1$ | $\mathbf{-32.9 \pm 0.3}$ | $4.9 \pm 0.4$ |
| DP4 | $6.0 \pm 0.4$ | $34.3 \pm 0.6$ | $2.92 \pm 0.05$ | $-31.6 \pm 0.4$ | $-11.2 \pm 0.2$ | $\mathbf{-20.4 \pm 0.2}$ |

[a] The prevailing contribution (enthalpic or entropic) is in bold.

attributed mainly to hydrogen bonding and protonation. It is usually considered that the intermolecular complexation between polyphenols and proline-rich proteins involves both hydrophobic interactions (*31*) and hydrogen bonding (*5*, *10*, *32*). On the one hand, hydrophobic interactions[mainly an entropic effect, as described by Israelachvili (*33*)], which are relatively long-range, depend on the respective solubility of both the protein and the polyphenol in the solvent. On the other hand, the formation of hydrogen bonds is considered to occur mainly between the hydrogen atom of the polyphenol acidic hydroxyl groups and the peptide bond H-acceptor sites. This short-range phenomenon tends to stabilize the complex formed (*34*).

Results given in **Table 2** were obtained using the one-site model, after removal of the first points, which tends to overestimate the enthalpic contribution and thus underestimate the entropic one. This overestimation is not too important in the case of monomers, but is not negligible in the case of DP4.

Therefore, we can assume that in the case of monomers, enthalpic phenomena overcome entropic ones (−25.1 vs −0.9 kJ $mol^{-1}$ for EgcG, −32.9 vs 4.9 kJ $mol^{-1}$ for EcG), whereas the opposite occurs with DP4 (−11.2 vs −20.4 kJ $mol^{-1}$).

**Stoichiometry.** Poly(L-proline) has an average degree of polymerization of 71, which means that if the flavanol/poly(L-proline) stoichiometry is measured to be 9 and 7 (monomers) or 6 (DP4) (**Table 2**), 1 proline unit of about 8 or 10 (monomers) or 12 (DP4) interacts with EgcG, EcG, or DP4. In all cases, the number of flavanol molecules bound on each poly(L-proline) is relatively low if we compare it with the number of residues per turn (three). This is probably due to steric hindrance phenomena. The difference observed between the monomers and the oligomer fraction may be explained (i) by the fact that the DP4 tannin has several binding sites and some tannin molecules may bind the poly(L-proline) through two flavan-3-ol units and/or (ii) by steric hindrance phenomena.

**Binding-Aggregation Scenario.** In a previous paper (*9*), we described an interaction mechanism consisting of successive stages, corresponding to (i) ligand binding and saturation of the PRP binding sites associated with very weak scattered light intensity and large particles (i.e., presence of very few particles), (ii) formation of flavan-3-ol-protein aggregates of relatively small size, and (iii) precipitation upon further flavan-3-ol addition. In the second stage, and for flavan-3-ol/poly(L-proline) ratios up to 17 and 20 (EcG and EgcG, respectively), relatively homogeneous particles (average size <50 nm) were observed. When the molar ratio was further increased above 20, increases of both the aggregate size and the polydispersity indices of the suspension were observed for EcG and EgcG, leading eventually to phase separation at the end of the experiment (molar ratios of 27 and 33, respectively). The data obtained by ITC highlight the fact that the formation of large aggregates requires much more ligand than the simple saturation of binding sites (usually for molar ratios between 6 and 9), as already observed by Pascal et al. on human proline-rich proteins interacting with EgcG (*27*).

We worked here with a simple system and model molecules. However, tannins in wine are a complex mixture of native and so-called derived tannins (e.g., oxidized tannins, flavanol-anthocyanins adducts), and these molecules may display a more complex behavior when interacting with proteins. Our next aim is to isolate these species to compare them with native flavanols.

## ABBREVIATIONS USED

PRP, proline-rich protein; Cat, catechin; Ec, epicatechin; EcG, epicatechin gallate; EgcG, epigallocatechin gallate; DP, degree of polymerization; ITC, isothermal titration calorimetry; DLS, dynamic light scattering.

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