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Influence of Wine Pectic Polysaccharides on the Interactions between Condensed Tannins and Salivary Proteins

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α-Amylase, a major human salivary protein, and IB8c, a representative of the proline-rich proteins, were obtained by isolation from saliva and by solid-phase synthesis, respectively. The interactions between these proteins and condensed tannins isolated from grape seeds were studied at different protein and tannin concentrations by measuring their aggregation. Pectic polysaccharides were isolated from wine, and their effect on protein tannin aggregation was assessed. The results presented in this study showed that the most acidic fractions of arabinogalactan proteins have the ability to inhibit the formation of aggregates between the grape seed tannins and the two different salivary proteins. Rhamnogalacturonan II has the same ability toward α-amylase but not IB8c under the conditions of the present study. Polysaccharides show effects at concentrations at which they are present in wine, which could mean an influence in wine astringency. The interaction between condensed tannins and α-amylase is differently affected by ionic strength when compared with IB8c.

KEYWORDS: Wine polysaccharides; condensed tannins; human salivary proteins; protein−tannin interaction, ionic strength

INTRODUCTION

Astringency is generally believed to be due to the binding of tannins present in beverages and food of plant origin with salivary proteins forming aggregates that lead to a lack in lubrication and to the characteristic dry and rough feeling (*1*). Tannins were shown to reduce saliva lubricating ability both by increasing friction and by reducing its viscosity (*2*), and studies have shown that provide evidence that the interaction between tannin and salivary proteins is indeed involved in the mechanism of astringency (*3*, *4*).

Several salivary proteins have been reported to interact with tannins: salivary α-amylase is inhibited (*5*, *6*) and precipitated by tannins (*7*), salivary histatins were shown to be able to precipitate condensed tannins (*8*−*10*), and all classes of salivary PRPs (proline-rich proteins) were shown to have high binding affinities for tannins (*11*). PRPs represent almost 70% of human parotid saliva. They can be divided in three groups: acidic, basic, and glycosilated PRPs. Amylase comprises most of the remainder of the total protein content of parotid saliva (*12*).

Although tannins seem to be effective in interacting with several salivary proteins, a study by Sarni-Manchado et al. (*13*) showed that two types of proteins of low molecular weight, PRPs and supposedly histatins, were more easily precipitated, even at low tannin concentrations. Another study showed that basic PRPs bind more effectively to condensed tannins than acidic, glycosylated PRPs, and gelatin (*14*). It is believed that the function of basic PRPs is to bind tannins to offer protection from its deleterious effects.

Protein−tannin interactions appear to be inhibited by the presence of some polysaccharides in solution (*15*−*19*). In fact, this was proposed as one of the possible mechanisms for the loss of astringency in ripening fruits: as the cellular structure softens during fruit ripening, there is an increase in water-soluble pectin fragments that could prevent the formation of aggregates between tannins and proteins in the mouth, leading to a modified astringency response (*19*). Other studies showed that the astringency of tannins is reduced by the addition of soluble pectin (*20*). This is related to the formation of complexes between pectin and tannins (*21*).

Two mechanisms have been proposed to explain how polysaccharides could inhibit protein−tannin interactions (*17*, *22*): (i) polysaccharides could encapsulate polyphenols interfering with their ability to bind proteins or (ii) some polysaccharides are polyelectrolytes and as such could form ternary complexes with the protein−polyphenol aggregate, enhancing its solubility in aqueous medium.

Previous studies have shown that ionic polysaccharides such as pectin, gum arabic, and polygalacturonic acid were able to prevent protein−tannin aggregation (*16*, *17*). Polysaccharides that are able to develop gel-like structures such as xanthan gum

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or hydrophobic pockets such as cyclodextrin were also shown to be capable of preventing protein−tannin associations (*16*, *19*).

Astringency is a fundamental sensation for a wine, particularly red wines that are rich in polyphenols, mainly from grape seeds and skins. It is acknowledged that high-quality red wines are balanced wines that have neither too much astringency, which would lead to a hard wine, nor too little, which would lead to an uninteresting and flat wine. Wines have polysaccharides that could affect the mouth feel properties. Major wine polysaccharides are arabinogalactan proteins (AGP) and rhamnogalacturonan II (RGII), pectic polysaccharides that originate from grape cell walls and mannoproteins (MP) that are produced by yeast during fermentation (*23*).

In a study by Vidal et al. (*24*), it was reported that both AGPs and RGII increase the fullness sensation of a model wine in the absence of procyanidins, while RGII significantly decrease the astringent attributes of this model solution. Moreover, in the presence of procyanidins, astringency descriptors were shown to decrease in the presence of RGII, while MPs and AGPs decreased bitterness (*25*).

Riou et al. (*26*) have studied the ability of several wine polysaccharides to inhibit tannin self-aggregation. They have found that the most neutral fraction of AGP had no impact on tannin aggregation, but that this self-aggregation was strongly inhibited by the most acidic AGP. RGII seemed to induce tannin aggregation, probably due to a co-aggregation between polysaccharide and tannin particles.

In this work, the influence of four isolated wine polysaccharides on the interactions between two different salivary fractions and a grape seed procyanidin fraction was studied in order to obtain further insights on the influence of wine polysaccharides on salivary protein−grape seed tannin interactions at the origin of astringency sensations.

Several studies focus on the study of protein−tannin interactions at a molecular level (*27*−*29*). However, in the present study, the experimental procedure measures the intensity of light scattered by aggregates (soluble or insoluble) of protein and tannin complexes, and how these "macroscopic" interactions are affected by wine polysaccharides.

The salivary proteins used were α-amylase, a globular protein very abundant in saliva, and a protein representative of basic PRPs that are supposed to adopt an elongated structure (type II helix), as shown for IB7 (*30*). These PRPs are known to have a high propensity to bind tannins. The PRP chosen was IB8c, a 61 amino acid peptide of which about 41% are proline, 23% are glycine, and 15% are glutamine residues. On the other hand, α-amylase has few proline residues (4%) and is a much larger protein (about 560 residues). IB8c was found in human saliva (*31*, *32*), and contains a repeat pattern that is recurring in several other human basic PRPs. In fact basic PRPs have considerable sequence homology and appear to result from post-translation proteolytic degradation of limited products (*31*). Charlton et al. (*33*) suggest that these multiple tandem repeats that can be seen in salivary PRPs could give increased length to the protein, allowing it to wrap around the tannin, thereby increasing the association by cooperative binding.

EXPERIMENTAL PROCEDURES

Grape Seed Tannins Isolation. Condensed tannins were extracted from *Vitis vinifera* grape seeds using an ethanol/water/chloroform solution (1:1:2, v/v/v). The 50% aqueous ethanol upper layer contained the polyphenols and was separated from the chloroform layer. Ethanol was removed using a rotator evaporator, and the resulting aqueous solution was extracted with ethyl acetate followed by precipitation with hexane in order to obtain the procyanidin oligomers, according to the procedure described in the literature (*34*).

The grape seed extract was fractionated through a TSK Toyopearl HW-40(s) gel column (100 × 10 mm i.d., with methanol 0.8 mL·min^{-1} as eluent) according to a procedure described in the literature with small modifications (*35*). After removing the lower molecular weight procyanidins with methanol 99.8% (v/v), the more polymerized procyanidins were obtained after elution with methanol/5% acetic acid (v/v), mixed with deionized water, and freeze-dried. This fraction was analyzed by liquid secondary ion mass spectrometry (LSIMS) on a VG Autospec EQ spectrometer as described in the literature (*35*). The fraction was shown to contain essentially tetramers, digallate ($[M + H]^+ = 1459$), and pentamers, gallate ($[M + H]^+ = 1595$), with a high degree of purity (> 98%). This fraction was chosen since it was shown to be a very reactive procyanidin fraction toward proteins (*15*).

Wine Polysaccharides Isolation and Purification. Five liters of white wine (Quinta de Bons Ares, Ramos Pinto, Portugal) was evaporated under reduced pressure at 30 °C to give a volume of 300 mL. Wine macromolecules were precipitated by adding 1.5 L of ethanol containing 40 mL of 37% HCl to the wine concentrate. After 48 h at 4 °C, the resulting solid was recovered, dissolved in water, and dialyzed extensively against water. The resulting solution was subjected to successive liquid chromatographies to retrieve wine polysaccharides as described in the literature (*36*). Elution profiles were followed with a refractive index and a UV detector (254 nm). In short, wine macromolecules were fractionated by ion exchange chromatography on a DEAE−Sephacel column equilibrated with 50 mM acetate buffer, pH 4.8, at a flow rate of 0.3 mL·min-1 . Four fractions were obtained by eluting with different concentrations of NaCl: 0, 50, 150, and 250 mM. These fractions were subjected to affinity chromatography to eliminate mannoproteins on a concanavalin-A column, with a buffer solution of 20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$. The unbound grape polysaccharides were recovered, and a size exclusion chromatography column of Sephacryl S-400HR was used to further purify these polysaccharides.

All the fractions were dialyzed extensively to remove all salts and freeze-dried. The glycosyl residue composition of each polysaccharide fraction was determined by GC-EI-MS (gas chromatography electron impact mass spectrometry) analysis of the trimethylsilyl methyl glycoside derivatives as described by Doco et al. (*37*).

Salivary Proteins. *IB8c Synthesis and Purification.* IB8c was chemically synthesized according to the sequence: SPPGKPQGPPPQGGNQPQGPPPPGKPQGPPPQGGNKPQGPPPPGKPQGPPPQGGSKSRSA. This synthesis was performed on an Applied Biosystems peptide synthesizer 433A (PE Biosystem, Courtabouef France) using F_{moc} strategy, in a similar way as described for IB7 by Simon et al. (*30*), with some modifications: the synthesis was made in single coupling mode, with 0.1 mmol of resin (Fmoc-Ala-NovasynTGA) and a 10-fold excess of amino acid.

The product was purified by semipreparative reversed-phase liquid chromatography (Waters 2487 dual-absorbance detector and 2695 separation module) using a C_{18} Waters Delta pack column (15 μm, 100 Å, 7.8 × 300 mm). The elution was carried out with 0.1% aqueous TFA (eluent A) and 0.1% TFA in acetonitrile (eluent B). The flow rate used was 3 mL·min^{-1} . The gradient that allowed the better purification was a linear change from 15% to 20% of B in 25 min. Matrix-assisted laser desorption ionization and time-of-flight (MALDI-TOF) mass spectrometry was performed on the crude mixture and on the final purified product after freeze-drying and dissolution in water. The spectra are shown in **Figure 1**. The purification allowed to obtain a product that was enriched in peptide IB8c with some contamination of truncated peptides. This contamination does not seem to be important because the fragments were very similar to IB8c.

α-Amylase Purification. α-Amylase was isolated as described in the literature (*38*). Briefly, α-amylase was isolated from human saliva after precipitation with ammonium sulfate and chromatography in a DEAE-Sephadex A50 column. The unbound eluate was dialyzed, freeze-dried, and subjected to polyacrylamide gel electrophoresis. The electrophoretic pattern has shown that this fraction is rich in α-amylase with negligible amounts of other proteins.

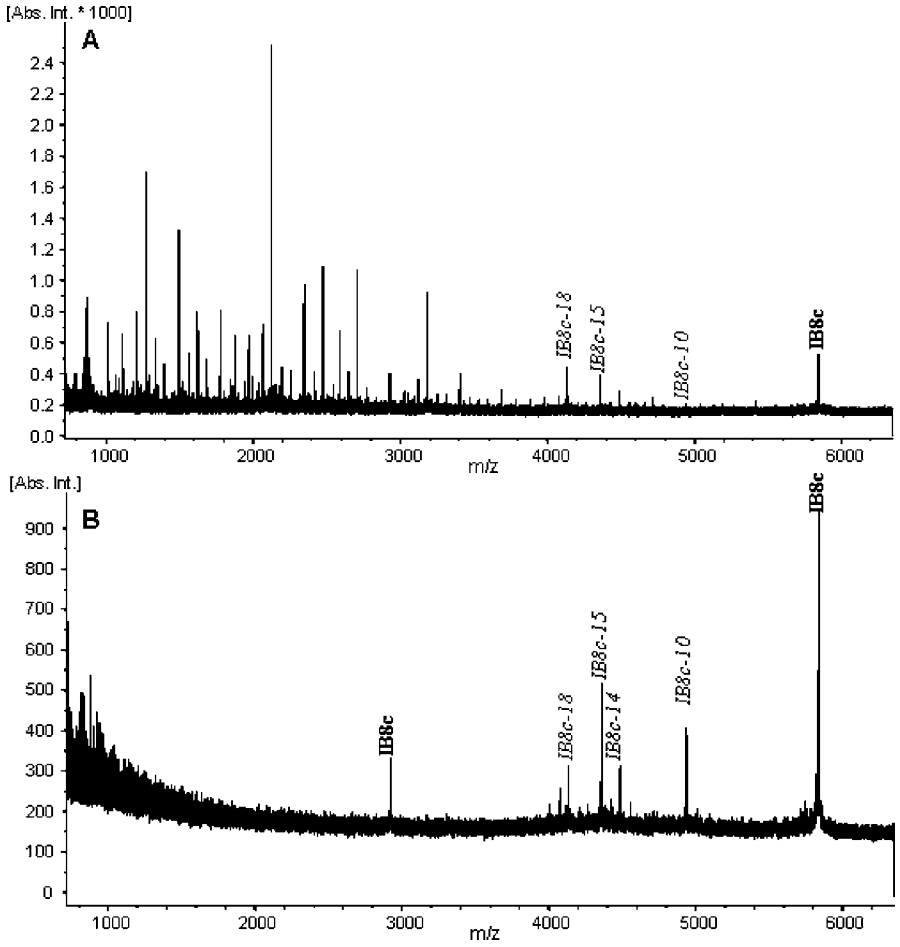

Figure 1. MALDI TOF spectrum of the crude synthesis product (**A**) and of the peptide after purification (**B**). The peaks labeled IB8c correspond to the $[M + H]^{1+}$ and $[M + 2H]^{2+}$ species, and the peaks labeled IB8c-X represent truncated peptides.

Light Scattering. A Perkin-Elmer LS 45 fluorimeter was used as a 90° light scattering photometer. Both the excitation and the emission wavelengths were set at 400 nm at which proteins, tannin, and polysaccharides do not absorb the incident light. A neutral density standard filter was used in some experiments to minimize detector overranging. Proteins (α-amylase and IB8c) and condensed tannins were allowed to interact at different concentrations until aggregates were formed, after which the light scattered by these aggregates was measured.

Stock solutions were prepared in 12% ethanol/water (v/v): a solution of 1.25 $g \cdot L^{-1}$ condensed tannin fraction, of 0.1 $g \cdot L^{-1}$ IB8c, of 0.44 $g \cdot L^{-1}$ α-amylase, and of 0.1 M acetate buffer, pH 5.0. All solutions were carefully filtered (0.45 μm) through nylon or cellulose acetate (protein solutions). After tentative approaches on protein and tannin concentrations, a final experiment was devised where several 2 mL microtubes were prepared with 4 μL of stock tannin solution, Then 16 μL of stock acetate buffer solution and different volumes of ethanol 12% solution were added. To each of these tubes, different volumes of the α-amylase solution were added between 2 and 128 μL. The final concentrations in each tube were 15.6 $mg \cdot L^{-1}$ tannin, 12% ethanol, and 5.0 mM acetate buffer (pH 5.0). The final volume of all the tubes was 320 μL. The tubes were shaken and left at room temperature for 30 min. After this time, the solutions were shaken and transferred to the fluorimeter cell where the intensity of light scattered was measured. Between each measurement, the cell was washed with 12% ethanol and rinsed with the solution to be measured. Next, several tubes were prepared under the same conditions but with a fixed α-amylase concentration of 22.0 $mg \cdot L^{-1}$ (the stoichiometric maximum), to which several volumes of tannin solution were added, to give final concentrations within the range 7.80 and 63.0 $mg \cdot L^{-1}$.

The same approach was used toward IB8c, but the concentrations used were different: a concentration of 31.2 $mg \cdot L^{-1}$ of tannin was tested with IB8c in the range 0.60−5.0 $mg \cdot L^{-1}$, and inversely, a concentration of 3.12 $mg \cdot L^{-1}$ (stoichiometric maximum) of IB8c was tested with tannin in a range of 19.5−46.8 $mg \cdot L^{-1}$.

The effect of ionic strength on aggregate formation between both proteins and tannin was tested by adding different volumes of a NaCl (0.50 M) solution to the microtubes containing tannin, 3.1 mM acetate buffer, pH 5.0, and 12% ethanol before protein addition. The effect of wine polysaccharides was tested by adding different volumes of polysaccharide solution to the microtube containing tannin, 3.1 mM acetate buffer, pH 5.0, and 12% ethanol, and after a 30 min rest at room temperature, the protein solution was added. All these experiments were prepared in at least triplicates, and the results are presented in mean ± standard deviation.

RESULTS AND DISCUSSION

Wine Polysaccharides Purification and Isolation. AGPs have been reported to share a core of structural features that consist in a ramified (1 → 3)-D-galactan inner core with (1 → 6)-linked galactan side chains that are highly substituted by arabinose and with minor amounts of rhamnose, xylose, and glucuronic acid (*39*). The analysis of the trimethylsilyl methyl glycoside derivatives of fractions eluted with 0, 50, and 150 mM NaCl from DEAE−Sephacel were shown to contain mostly arabinose, galactose, and glucuronic acid. They also contained small amounts of xylose and rhamnose (data not shown). This is the typical composition of AGPs; these fractions were named AGP0, AGP50, and AGP150, respectively. The quantities isolated of each of these polysaccharides were 27.0, 4.0, and 54.0 mg, respectively.

RGIIs have a backbone of at least eight residues of galacturonic acid with four different secondary side chains. RGII contains 12 different glycosyl residues that include galacturonic and glucuronic acids, rhamnose, galactose, arabinose, fucose, and several rare sugars that are diagnostic of RGII presence, such as apiose, aceric acid, 2-*O*-methyl fucose, 2-*O*-methyl xylose, Kdo (3-deoxyoctulosonic acid), and Dha (3-deoxy-D-*lyxo*-heptulosaric acid) (*40*, *41*). The fraction eluted with 250 mM NaCl has shown to contain a high concentration in uronic acids, rhamnose, arabinose, galactose, and fucose as well as the rare monosaccharides apiose, Kdo, Dha, and aceric acid. Therefore, this fraction was classified as RGII and yielded an amount of 61.0 mg.

Effect of Protein and Tannin Concentrations on Their Aggregation. The experiments in this study were all performed in 12% ethanol to mimic a model wine, and a pH of 5.0 was chosen as it has already been referred as a pH at which salivary proteins strongly interact with condensed tannins (*7*) and, on the other hand, correspond to a intermediary pH between wine pH (~3.4) and saliva pH (7.0). Indeed, salivary pH drops with ingestion of acidic drinks (*42*) and the degree of acidity in saliva depending on the sampled volume, buffering capacity, and mode of drinking.

As the concentration of ethanol in the mouth after sampling wine could vary with salivary flow rate, wine volume ingested, and with the initial ethanol content of the wine, a 12% concentration of ethanol was chosen as it is widely used to mimic model wines.

Since the reagents used were only available in small quantities, a fluorimeter was used as a sensitive light scattering apparatus to replace the conventional nephelometer (*7*, *43*). The excitation and emission monochromators of the fluorimeter were set for the same wavelength (400 nm) at which proteins, tannins, and polysaccharides do not absorb the incident light. Both monochromators are set at a 90° angle, and only the light that was scattered by the aggregates formed between proteins and tannins was measured.

Control experiments were performed with solutions of protein, tannin, and polysaccharide. No significant differences were observed from the light scattered by a 12% ethanol solution.

It is well-known that protein-tannin interactions are influenced by the relative concentrations of protein and tannin (*7*, *44*−*47*). Moreover, the type of aggregates formed between proteins and tannins depends on the relative concentrations of these components (*28*, *45*, *48*−*50*). At stoichiometric concentrations of tannin and proteins, polyphenols are able to act as multidentate ligands and bridge proteins or protein−tannin complexes, forming large aggregates that scatter more light. At lower or higher protein/tannin ratios, smaller particles are formed, resulting in lower light scattering.

In order to establish the stoichiometric concentrations of the two different proteins (α-amylase and IB8c) and condensed tannins, some experiments were made with different concentrations of protein and tannin in order to obtain the maximum aggregation in solution. First, a concentration of 15.6 mg·L^{-1} tannin (10.4 μM, considering an average molecular weight of 1500 g·mol^{-1} for the tannin fraction) was chosen and allowed to react with increasing concentrations of α-amylase (**Figure 2A**). It was observed that the increase in protein concentration led to an increase in aggregate formation, until a maximum (stoichiometric concentration) from which a further increase in α-amylase concentration did not change the light scattered by the aggregates. In order to confirm the stoichiometry, the concentration of α-amylase at this point (22.0 mg·L^{-1}, 0.4 μM considering an average molecular weight of 56000 g·mol^{-1}) was chosen and allowed to react with increasing concentrations of the tannin fraction (**Figure 2B**). A similar behavior was observed: there was a rapid increase in aggregate formation with an increase in tannin concentration reaching a point from which the light scattered intensity remained constant even with a further increase in tannin concentration. As expected, the concentration of tannin at which the light scattered intensity remained steady was the same concentration used another graph (**Figure 2A**). This stoichiometric concentration corresponds to the maximum of light scattering resulting from the maximum aggregation with no excess of protein or tannin.

Charlton has proposed a constant to describe tannin−protein interactions. By fitting the experimental data with the equation proposed (*29*, *48*), this constant was calculated and was estimated around 0.5 μM with a tannin/α-amylase molar ratio close to 25.

Following this, a similar study was made for IB8c. Increasing concentrations of IB8c were added to a solution containing the same concentration of tannin used in **Figure 2A** (15.6 mg·L^{-1}). However, it was observed that the intensity of the light scattered by the aggregates was low and practically did not change by varying protein concentrations (data not shown). Thus, a twofold higher concentration of tannin (31.2 mg·L^{-1}, 20.8 μM) was assayed yielding better results (**Figure 2C**). The formation of aggregates increased simultaneously with the concentration of IB8c. Initially there was a rapid increase in the light scattered by protein tannin aggregates with increases in IB8c concentration, followed by a slower increase, reaching a maximum concentration of IB8c (3.12 mg·L^{-1}, 0.5 μM considering a molecular weight of 5843 g.mol^{-1}). With higher IB8c concentrations, a slight decrease of the light scattered was observed. This latter phenomenon has been observed with other proteins such as BSA (*16*) or gelatin (*46*), and it can be explained by the proposed mechanism of aggregate formation when the protein is in excess, polyphenol binding sites would be occupied by protein molecules and it would be very unlikely that bridging would occur between protein-tannin complexes thereby, resulting in smaller aggregates (*46*, *49*).

The concentration of 3.12 mg·L^{-1} for IB8c was chosen since it was near maximum aggregation. In order to confirm this maximum, the influence of increasing tannin concentration on aggregate formation was measured using this concentration (**Figure 2D**). We observed that the light scattered intensity increased concomitantly with tannin concentration up to a plateau corresponding to a tannin concentration of approximately 31.2 mg·L^{-1}. This was the same procyanidin concentration used in **Figure 2C**, thus confirming the stoichiometry. The constant defined by Charlton mentioned before was estimated to be 0.6 μM, and the tannin/IB8c molar ratio was 35.

Interestingly, the small PRP protein (61 amino acids) binds more tannins than the large α-amylase (around 560 residues). This may be accounted for by the 3D structure of both proteins: α-amylase is globular and offers only its external surface to bind tannins whereas IB8c probably adopts a type II helix, as it has been demonstrated for its parent protein IB7 (*30*). It may therefore offer more interacting contact points.

In fact basic PRPs have been referred as "tannin sponges" (*29*), and Charlton et al. (*31*) explained this phenomenon by the fact that the presence of multiple repeat regions rich in proline provide rigid regions favorable for tannin binding, and flexible hinges on the protein allows it to fold and "wrap around" the tannin, thereby increasing the association by cooperative

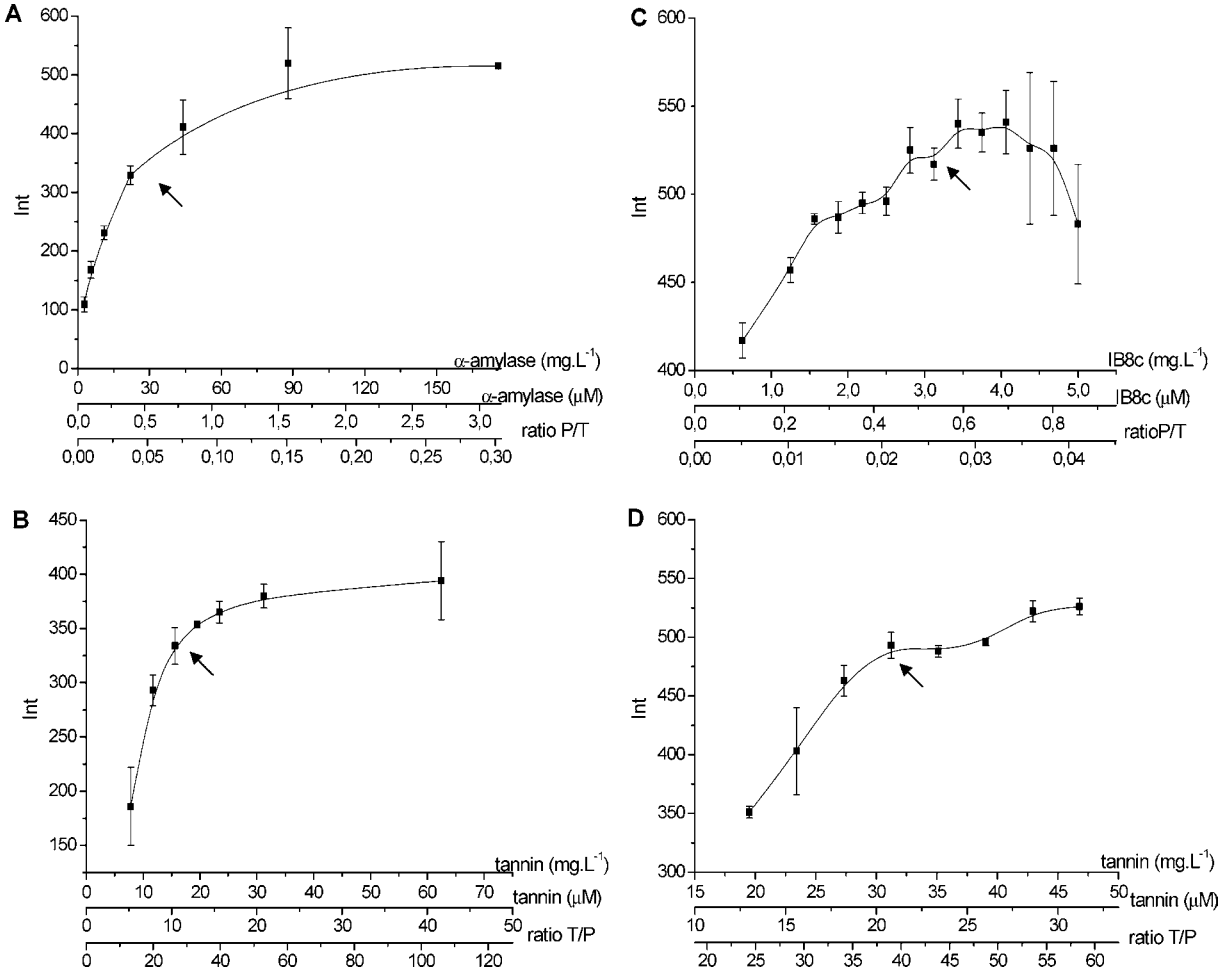

Figure 2. Influence of protein concentration on aggregate formation with procyanidins 15.6 (**A**) and 31.2 mg·L^{-1} (**C**). Influence of tannin concentration on the aggregate formation with *a*-amylase 22.0 mg·L^{-1} (**B**) and Ib8c 3.12 mg·L^{-1} (**D**). The arrows point to the mentioned concentrations. The lower scales represent the concentrations in μM (calculated using average molecular weights of 1500 g·mol^{-1} for tannin, 5843 g·mol^{-1} for IB8c, and 56000 g·mol^{-1} for α-amylase), and the molar ratio protein/tannin (P/T) or tannin/protein (T/P).

intramolecular interactions. The fact that α-amylase exhibits also good association with tannin could be explained by its 10-fold higher size.

Influence of Ionic Strength on Aggregate Formation. The influence of ionic strength on aggregate formation between proteins and tannins at the concentrations mentioned above was studied (**Figure 3**). Control experiments showed that the presence of NaCl did not induce protein or tannin aggregation at the concentrations used. The initial ionic strength was 2.0 mM, corresponding to an acetate buffer concentration of 3.1 mM.

A small inhibitory effect on the aggregation between α-amylase and tannin was observed when the ionic strength was increased. Oppositely, for IB8c it was observed a significant increase in the light scattered intensity with the increase in ionic strength.

α-Amylase and IB8c have calculated isoelectric points of 6.34 and 11.39, respectively (Swiss-Prot codes P04745 and P02812, respectively), suggesting that they are probably positively charged at pH 5. The fact that the isoelectric point of α-amylase is much nearer the pH of this study, and thus relatively less charged, could explain the fact that it showed a smaller effect with increasing ionic strength. Condensed tannins are weak acids with a pKa of 9−10 (*36*). The increase in aggregation between tannin and IB8c at higher ionic strength could be explained by

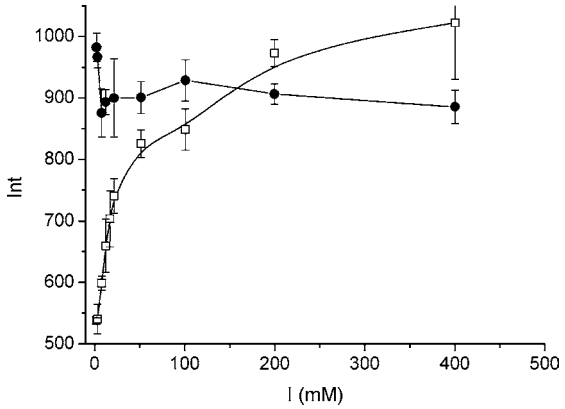

Figure 3. Influence of ionic strength on aggregate formation between grape seed procyanidins 15.6 mg·L^{-1} and *a*-amylase 22.0 mg·L^{-1}(●) and between grape seed procyanidins 31.2 mg·L^{-1} and Ib8c 3.12 mg·L^{-1} (□) at 12% ethanol, 3.1 mM acetate buffer, pH 5.0. Ionic strength (*I*) was set using NaCl.

ion hydration of salts removing the water and ethanol from the bulk of the aggregate structure promoting protein and tannin interactions. Several authors (*27*, *48*, *50*) have focused on the interaction between proline-rich peptides and polyphenols and suggested that the binding of protein to polyphenol is essentially

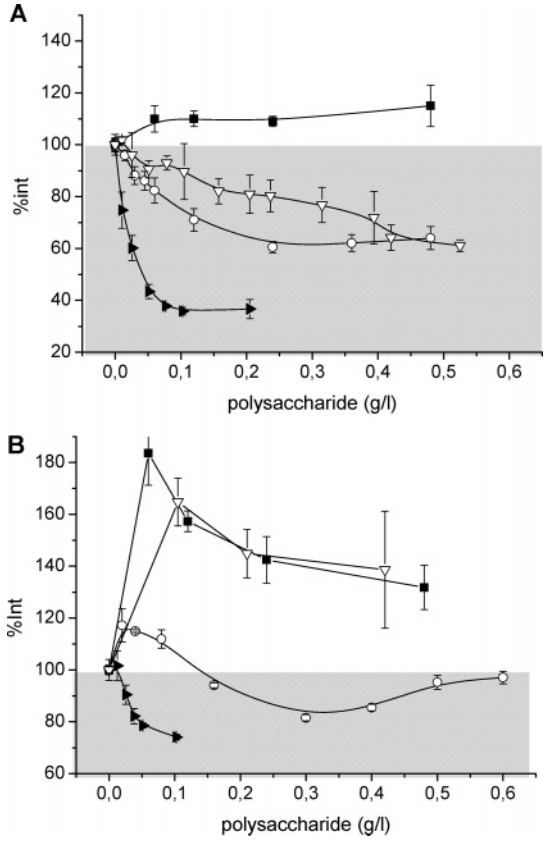

Figure 4. Influence of polysaccharide concentration on aggregate formation between procyanidins 15.6 mg·L^{-1} and α-amylase 22.0 mg·L^{-1} (**A**) and between procyanidins 31.2 mg·L^{-1} and IB8c 3.1 mg·L^{-1} (**B**) at 12% ethanol, 3.1 mM acetate buffer, pH 5.0, given in percentage (where 100% refers to intensity at zero polysaccharide concentration). The areas below 100% were shadowed for better visualization. ■, AGP0; ○, AGP50; ▲, AGP150; and ▽, RGII.

hydrophobically driven, with the aromatic polyphenol rings stacking against proline residues side chains.

However, these studies were performed in the presence of organic solvents. Working in hydroalchoolic medium, at pH 3.5, Simon et al. (*29*) presented evidence that the interaction between a proline-rich peptide and condensed tannins occurred in the hydrophilic side of the peptide and did not observe hydrophobic stacking.

The type of interactions between tannins and proteins appear to be dependent on the type of tannin (*51*) and protein (*52*). The fact that IB8c and α-amylase have different behaviors with increasing ionic strengths on the interactions with the same condensed tannin fraction suggests that these two different proteins might interact with tannins differently. This seems plausible since these proteins have different characteristics: α-amylase has a globular structure and is about 10 times bigger than IB8c, which has a more extended structure.

Effect of Wine Polysaccharides on the Interaction between Salivary Proteins and Tannin. The influence of four polysaccharide fractions isolated from wine on the interactions between proteins and tannins were tested (**Figure 4**). In order to mimic the situation in the mouth where tannins and polysaccharides simultaneously contact with salivary proteins, a solution of tannins−polysaccharides was prepared to which the different salivary proteins were subsequently added.

However, the concentration of tannins and protein during experiments are much lower than it was expected to found in wine (1−4 g·L^{-1} (*53*)) and mouth (3 g·L^{-1} (*54*)), and their concentration in the mouth depends on the salivary flow rate, ingested volume, and saliva characteristics. the time the wine is allowed to be in the mouth and different wine characteristics (lower or higher polyphenol levels). The range of concentrations was chosen to be within the range of the apparatus and to avoid the formation of cloudiness: keeping the amount of dispersed matter small, the absorption is negligible and the intensity of scattered light is proportional to the concentration of the dispersed phase.

The interactions between proteins and tannins at the concentrations previously established were assessed in the presence of increasing concentrations of polysaccharide. The results were presented in percentage of the measured intensity (%Int) in order to be able to compare results more easily: the light scattered by the solutions containing protein and tannins only was considered to be 100%; values lower than 100% indicate less scattered light that may be due to less aggregates or, which is more probable, smaller aggregates, however this would require confirmation.

Controls showed that tannin/carbohydrate solutions did not induce aggregate formation, nor did IB8c/carbohydrate solutions. There was some light scattering in solutions of one of the wine polysaccharides (RGII) with α-amylase, but this effect was relatively low when compared with total intensity of the α-amylase-tannin solution at the lowest RGII concentration (about 20%) and decreased with increase in RGII concentration.

In the case of α-amylase, it was observed that AGP50, AGP150, and RGII were able to prevent protein−tannin aggregation: there is a rapid decrease in the light scattered intensity with the increase in polysaccharide concentration (**Figure 4**). From this point of maximum inhibition, a further increase in polysaccharide concentration does not lead to any change in light scattering intensity. AGP150 appears to be more efficient than the other polysaccharides used since it was leading to less light scattering at lower concentrations, suggesting the presence of smaller aggregates. Both RGII and AGP50 were able to prevent aggregation at similar concentrations, but AGP50 seemed to be slightly more efficient. AGP0 appears to have no reducing effect on the aggregate formation between α-amylase and condensed tannins under these conditions. In fact, there seemed to be a slight increase in the intensity of light scattered in the presence of AGP0.

For IB8c, a similar behavior was observed with AGP150 proving to be very effective inhibiting IB8c-condensed tannin aggregate formation. On the other hand, AGP50 showed a somewhat sinuous behavior: at low concentrations there was an increase in aggregate formation. With increasing concentration of AGP50 aggregate formation decreases and with further increase the percentage returned to the initial value of 100%. Apparently, this polysaccharide is more effective preventing aggregate formation between IB8c and this condensed tannin fraction at a limited range of AGP50 concentrations, and this range of concentrations is higher than those usually present in wine. In the presence of AGP0 and RGII there was an increase in the aggregate formation.

The isolation of wine polysaccharides was performed according to the method described by Vernhet et al. (*36*). The authors observed that the polysaccharides were negatively charged and that their charge densities were related to their order of elution from DEAE−Sephacel and to their uronic acid content (the most charged being the one richer in uronic acids and the one eluted from the ionic exchange column at higher ionic

strength). It seems safe to assume that the polysaccharides isolated in this study would follow the same sequence in decreasing order of magnitude of charge densities: AGP150 > AGP50 > AGP0. In the same study, RGII is reported to have the highest negative charge density of all the grape polysaccharides.

It is interesting to note that the inhibition of aggregate formation between both proteins and condensed tannins by the AGP fractions seems to be related to their charges: the most effective AGP is the one that has the stronger ionic character. Vernhet et al. (*36*) have proposed that wine polysaccharides would likely establish electrostatic and ionic interactions with other compounds or particles such as proteins. Therefore, AGP prevention of protein−tannin aggregation could be related to their ionic characteristics.

An acidic fraction of AGP has been shown to prevent tannin self-aggregation (*26*), this was proposed to occur by adsorption of polysaccharide to particles already formed by tannin self-aggregation preventing them to grow further. The charged AGP fractions could either prevent the formation of protein−tannin aggregates by preventing polyphenols from associating with proteins, or they could inhibit the formation of large protein−tannin aggregates by forming ternary complexes with proteins and polyphenols as proposed elsewhere (*17*, *22*).

The most neutral fraction of AGP (AGP0) seemed to have the reverse effect on the aggregation between protein and tannin: under these conditions, in the presence of AGP0 there is an increase in the light scattered by the protein−tannin aggregates. This effect is relatively small on the case of α-amylase and very evident in the case of IB8c. This could be due to a co-aggregation of AGP0 with protein and tannin complexes, forming larger aggregates, as previously suggested for the neutral polysaccharide dextran (*16*).

RGII has shown different behaviors with α-amylase and IB8c: it has been relatively effective in preventing aggregate formation between α-amylase and tannin but favored the formation of aggregates between IB8c and tannin. Since there was some aggregation between α-amylase and RGII observed in the control experiments, it could be supposed that RGII prevents tannin to access α-amylase by binding to the protein.

For the lower concentrations of all the polysaccharides tested, an increase in aggregation relatively to IB8c−tannin alone was observed. Polysaccharides could bind to tannin or protein−tannin aggregates, without being able to prevent further cross-linking at these lower concentrations, but increasing the size of the aggregates by their presence.

In conclusion, it was observed that α-amylase and IB8c interact with tannins forming aggregates, but that this aggregation is differently affected by ionic strength and by wine polysaccharides. α-Amylase aggregation with tannins is slightly reduced when ionic strength is increased, while IB8c aggregation with tannins is greatly favored, which suggests different mechanisms of aggregation. The most acidic fractions of wine AGPs have inhibited the aggregation between both proteins and tannins. AGPs are present in red wines in concentrations of about 100−200 mg·L^{-1} and RGII of 100−150 mg·L^{-1} (*23*). In this study, these polysaccharides have shown some degree of inhibitory effects at concentrations as low as 20 mg·L^{-1} (AGP150 for both proteins and AGP50 and RGII for α-amylase). This could mean that wines with high levels of these polysaccharides would present lower astringency, even if they had high polyphenolic content. However, some polysaccharides have shown the reverse effect (AGP0 and RGII in the case of IB8c), which could mean that their presence could contribute to an increased astringency. It should be noted however that these interactions depend not only on the two proteins studied but also on the overall proteins present in saliva such as mucins, histatins, and others that could contribute to this sensation. Care should be taken when extrapolating this results to in-mouth astringency. On the other hand, these interactions may be affected by several factors, namely, ionic strength, pH, or temperature. However, other studies including all salivary proteins have already shown that wine polysaccharides do decrease the astringency and bitterness of procyanidins (*25*).

ABBREVIATIONS USED

PRP, proline-rich protein; AGP, arabinogalactan protein; RGII, rhamnogalacturonan II.

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LITERATURE CITED

(1) Green, B. G. Oral astringency—a tactile component of flavor. *Acta Psychol.* **1993**, *84*, 119−125.
(2) Prinz, J. F.; Lucas, P. W. Saliva tannin interactions. *J. Oral Rehabil.* **2000**, *27*, 991−994.
(3) Kallithraka, S.; Bakker, J.; Clifford, M. N. Evidence that salivary proteins are involved in astringency. *J. Sens. Stud.* **1998**, *13*, 29−43.
(4) Horne, J.; Hayes, J.; Lawless, H. T. Turbidity as a measure of salivary protein reactions with astringent substances. *Chem. Senses* **2002**, *27*, 653−659.
(5) McDougall, G. J.; Shpiro, F.; Dobson, P.; Smith, P.; Blake, A.; Stewart, D. Different polyphenolic components of soft fruits inhibit α-amylase and α-glucosidase. *J. Agric. Food Chem.* **2005**, *53*, 2760−2766.
(6) Kandra, L.; Gyemant, G.; Zajacz, A.; Batta, G. Inhibitory effects of tannin on human salivary α-amylase. *Biochem. Biophys. Res. Commun.* **2004**, *319*, 1265−1271.
(7) de Freitas, V.; Mateus, N. Nephelometric study of salivary protein−tannin aggregates. *J. Sci. Food Agric.* **2002**, *82*, 113−119.
(8) Naurato, N.; Wong, P.; Lu, Y.; Wroblewski, K.; Bennick, A. Interaction of tannin with human salivary histatins. *J. Agric. Food Chem.* **1999**, *47*, 2229−2234.
(9) Wroblewski, K.; Muhandiram, R.; Chakrabartty, A.; Bennick, A. The molecular interaction of human salivary histatins with polyphenolic compounds. *Eur. J. Biochem.* **2001**, *268*, 4384−4397.
(10) Yan, Q. Y.; Bennick, A. Identification of histatins as tannin-binding proteins in human saliva. *Biochem. J.* **1995**, *311*, 341−347.
(11) Bacon, J. R.; Rhodes, M. J. C. Binding affinity of hydrolyzable tannins to parotid saliva and to proline-rich proteins derived from it. *J. Agric. Food Chem.* **2000**, *48*, 838−843.
(12) Dodds, M. W. J.; Johnson, D. A.; Yeh, C. K. Health benefits of saliva: a review. *J. Dent.* **2005**, *33*, 223−233.

(13) Sarni-Manchado, P.; Cheynier, V.; Moutounet, M. Interactions of grape seed tannins with salivary proteins. *J. Agric. Food Chem.* **1999**, *47*, 42−47.
(14) Lu, Y.; Bennick, A. Interaction of tannin with human salivary proline-rich proteins. *Arch. Oral Biol.* **1998**, *43*, 717−728.
(15) Carvalho, E.; Póvoas, M.; Mateus, N.; De Freitas, V. Application of flow nephelometry to the analysis of the influence of carbohydrates on protein-tannin interactions. *J. Sci. Food Agric.* **2006**, *86*, 891−896.
(16) de Freitas, V.; Carvalho, E.; Mateus, N. Study of carbohydrate influence on protein-tannin aggregation by nephelometry. *Food Chem.* **2003**, *81*, 503−509.
(17) Luck, G.; Liao, H.; Murray, N. J.; Grimmer, H. R.; Warminski, E. E.; Williamson, M. P.; Lilley, T. H.; Haslam, E. Polyphenols, astringency and proline-rich proteins. *Phytochemistry* **1994**, *37*, 357−371.
(18) Mateus, N.; Carvalho, E.; Luis, C.; de Freitas, V. Influence of the tannin structure on the disruption effect of carbohydrates on protein−tannin aggregates. *Anal. Chim. Acta* **2004**, *513*, 135−140.
(19) Ozawa, T.; Lilley, T. H.; Haslam, E. Polyphenol interactions—astringency and the loss of astringency in ripening fruit. *Phytochemistry* **1987**, *26*, 2937−2942.
(20) Taira, S.; Ono, M.; Matsumoto, N. Reduction of persimmon astringency by complex formation between pectin and tannins. *Postharvest Biol. Technol.* **1997**, *12*, 265−271.
(21) Hayashi, N.; Ujihara, T.; Kohata, K. Reduction of catechin astringency by the complexation of gallate-type catechins with pectin. *Biosci. Biotechnol. Biochem.* **2005**, *69*, 1306−1310.
(22) Haslam, E. *Pratical Polyphenolics: From Structure to Molecular Recognition and Physiological Action*; Cambridge University Press: 1998.
(23) Doco, T.; Williams, P.; Moutounet, M.; Pellerin, P. Les polysaccharides du vin. *Bull. OIV* **2000**, *73*, 785−792.
(24) Vidal, S.; Francis, L.; Williams, P.; Kwiatkowski, M.; Gawel, R.; Cheynier, W.; Waters, E. The mouth-feel properties of polysaccharides and anthocyanins in a wine like medium. *Food Chem.* **2004**, *85*, 519−525.
(25) Vidal, S.; Courcoux, P.; Francis, L.; Kwiatkowski, M.; Gawel, R.; Williams, P.; Waters, E.; Cheynier, V. Use of an experimental design approach for evaluation of key wine components on mouth-feel perception. *Food. Qual. Preferences* **2004**, *15*, 209−217.
(26) Riou, V.; Vernhet, A.; Doco, T.; Moutounet, M. Aggregation of grape seed tannins in model wine—effect of wine polysaccharides. *Food Hydrocolloids* **2002**, *16*, 17−23.
(27) Charlton, A. J.; Haslam, E.; Williamson, M. P. Multiple conformations of the proline-rich protein/epigallocatechin gallate complex determined by time- averaged nuclear overhauser effects. *J. Am. Chem. Soc.* **2002**, *124*, 9899−9905.
(28) Jobstl, E.; O'Connell, J.; Fairclough, J. P. A.; Williamson, M. P. Molecular model for astringency produced by polyphenol/protein interactions. *Biomacromolecules* **2004**, *5*, 942−949.
(29) Simon, C.; Barathieu, K.; Laguerre, M.; Schmitter, J. M.; Fouquet, E.; Pianet, I.; Dufourc, E. J. Three-dimensional structure and dynamics of wine tannin-saliva protein complexes. A multitechnique approach. *Biochemistry* **2003**, *42*, 10385−10395.
(30) Simon, C.; Pianet, I.; Dufourc, E. J. Synthesis and circular dichroism study of the human salivary proline-rich protein ib7. *J. Pept. Sci.* **2003**, *9*, 125−131.
(31) Kauffman, D. L.; Bennick, A.; Blum, M.; Keller, P. J. Basic proline-rich proteins from human parotid-saliva - relationships of the covalent structures of 10 proteins from a single individual. *Biochemistry* **1991**, *30*, 3351−3356.
(32) Messana, I.; Cabras, T.; Inzitari, R.; Lupi, A.; Zuppi, C.; Olmi, C.; Fadda, M. B.; Cordaro, M.; Giardina, B.; Castagnola, M. Characterization of the human salivary basic proline-rich protein complex by a proteomic approach. *J. Proteome Res.* **2004**, *3*, 792−800.
(33) Charlton, A. J.; Baxter, N. J.; Lilley, T. H.; Haslam, E.; McDonald, C. J.; Williamson, M. P. Tannin interactions with a full-length human salivary proline-rich protein display a stronger affinity than with single proline-rich repeats. *FEBS Lett.* **1996**, *382*, 289−292.
(34) Michaud, M. M. J.; Lagaze, P.; Masquelier, J. Fractionnement des oligomeres flavanoliques du raisin. *Bull. Soc. Pharm. Bordeaux* **1971**, *110*, 111−116.
(35) de Freitas, V. A. P.; Glories, Y.; Bourgeois, G.; Vitry, C. Characterisation of oligomeric and polymeric procyanidins from grape seeds by liquid secondary ion mass spectrometry. *Phytochemistry* **1998**, *49*, 1435−1441.
(36) Vernhet, A.; Pellerin, P.; Prieur, C.; Osmianski, J.; Moutounet, M. Charge properties of some grape and wine polysaccharide and polyphenolic fractions. *Am. J. Enol. Vitic.* **1996**, *47*, 25−30.
(37) Doco, T.; O'Neill, M. A.; Pellerin, P. Determination of the neutral and acidic glycosyl-residue compositions of plant polysaccharides by GC-EI-MS analysis of the trimethylsilyl methyl glycoside derivatives. *Carbohydr. Polym.* **2001**, *46*, 249−259.
(38) de Freitas, V.; Mateus, N. Structural features of procyanidin interactions with salivary proteins. *J. Agric. Food Chem.* **2001**, *49*, 940−945.
(39) Pellerin, P.; Vidal, S.; Williams, P.; Brillouet, J. M. Characterization of 5 type-ii arabinogalactan-protein fractions from red wine of increasing uronic-acid content. *Carbohydr. Res.* **1995**, *277*, 135−143.
(40) Pellerin, P.; Doco, T.; Vidal, S.; Williams, P.; Brillouet, J. M.; Oneill, M. A. Structural characterization of red wine rhamnogalacturonan II. *Carbohydr. Res.* **1996**, *290*, 183−197.
(41) O'Neill, M. A.; Ishii, T.; Albersheim, P.; Darvill, A. G. Rhamnogalacturonan ii: Structure and function of a borate cross-linked cell wall pectic polysaccharide. *Annu. Rev. Plant Biol.* **2004**, *55*, 109−139.
(42) Johansson, A. K.; Lingstrom, P.; Imfeld, T.; Birkhed, D. Influence of drinking method on tooth-surface ph in relation to dental erosion. *Eur. J. Oral Sci.* **2004**, *112*, 484−489.
(43) Monteleone, E.; Condelli, N.; Dinnella, C.; Bertuccioli, M. Prediction of perceived astringency induced by phenolic compounds. *Food. Qual. Preferences* **2004**, *15*, 761−769.
(44) Carvalho, E.; Mateus, N.; de Freitas, V. Flow nephelometric analysis of protein−tannin interactions. *Anal. Chim. Acta* **2004**, *513*, 97−101.
(45) Poncet-Legrand, C.; Edelmann, A.; Putaux, J.-L.; Cartalade, D.; Sarni-Manchado, P.; Vernhet, A. Poly(l-proline) interactions with flavan-3-ols units: Influence of the molecular structure and the polyphenol/protein ratio. *Food Hydrocolloids* **2006**, *20*, 687−697.
(46) Siebert, K. J.; Troukhanova, N. V.; Lynn, P. Y. Nature of polyphenol-protein interactions. *J. Agric. Food Chem.* **1996**, *44*, 80−85.
(47) Hagerman, A. E.; Butler, L. G. Determination of protein in tannin-protein precipitates. *J. Agric. Food Chem.* **1980**, *28*, 944−947.
(48) 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.
(49) Siebert, K. J.; Lynn, P. Y. Effect of protein-polyphenol ratio on the size of haze particles. *J. Am. Soc. Brew. Chem.* **2000**, *58*, 117−123.
(50) Baxter, N. J.; Lilley, T. H.; Haslam, E.; Williamson, M. P. Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. *Biochemistry* **1997**, *36*, 5566−5577.

(51) 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.

(52) Artz, W. E.; Bishop, P. D.; Dunker, A. K.; Schanus, E. G.; Swanson, B. G. Interaction of synthetic proanthocyanidin dimer and trimer with bovine serum-albumin and purified bean globulin fraction g-1. *J. Agric. Food Chem.* **1987**, *35*, 417−421.

(53) Aps, J. K. M.; Martens, L. C. Review: The physiology of saliva and transfer of drugs into saliva. *Forensic Sci. Int.* **2005**, *150*, 119−131.

(54) Ribéreau-Gayon, P.; Yves Glories; Alain Maujean; Dubourdieu, D. *Traité d'œnologie tome 2: Chimie du vin. Stabilisation et traitements*; Paris, Dunod La Vigne: 2004.

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