JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Influence of the Glycosylation of Human Salivary Proline-Rich Proteins on Their Interactions with Condensed Tannins

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Binding of condensed tannins to salivary proteins is supposed to be involved in their astringency. First, complexes arising from the interaction of saliva from two individuals and tannins were studied. Then interaction mixture models containing purified saliva proteins were developed. The highest polymerized tannins predominantly precipitated together with the salivary proteins. Electrophoresis of proteins in combination with thiolysis analysis of tannins indicated proline-rich protein (PRP)−polyphenol complexes in precipitated fractions and also in the soluble ones with individual differences. Individual salivas exhibiting different protein patterns were discriminated with regard to their ability to interact with tannins. From binding studies with purified classes of salivary proteins, interactions were shown to depend on the nature of the protein, in particular on their glycosylation state. For low concentrations of tannins, glycosylated PRP−tannin interactions led to complexes that remained soluble, whereas those arising from nonglycosylated PRP−tannin interactions were precipitated. This finding could indicate that under physiological conditions, complexes involving glycosylated proteins maintain part of the lubrication of the oral cavity, whereas tannin trapping leads to a lower astringency perception.

KEYWORDS: Salivary proteins; polyphenols; astringency; interaction; proline-rich protein

INTRODUCTION

Plant polyphenols are secondary metabolites widely distributed in the higher plant kingdom. Thus, they are generally encountered in plant-derived foods and beverages. Polyphenols show a great diversity of structures and properties. Among them, condensed tannins (i.e., proanthocyanidins) are polymeric compounds in which monomeric units consist of flavan-3-ols linked by interflavan bonds from C4 of the upper unit to C8 and to a lesser extent C6 of the lower unit (B-type). Condensed tannins have attracted considerable interest because of their biological activities and organoleptic properties. They are believed to play a role in plant defense mechanisms against herbivorous ingestion due to their unpalatability and antinutritional properties (*1*). Underlying these phenomena is their distinctive ability to form intermolecular complexes with each other and with other molecules. It has been stated that their most predominant characteristic is their affinity for proteins. Thus, many of their actions appear to depend, either directly or indirectly, on this ability, which leads to the formation of soluble or insoluble complexes. In particular, the astringency of tannins, which affects their palatability, is reported to be related to the formation of complexes with salivary proteins, which may result in a decrease of saliva lubricating properties and greater friction on the mouth surface (*2*).

Saliva contains numerous proteins, and the most abundant are proline-rich proteins (PRPs), which constitute about 70% of the total content of parotid saliva (*3*). They are characterized by a predominance of proline (25−42%), glycine (16−22%), and glutamic/glutamine (15−28%) residues. The major glycosylated PRP consists of 57% protein and 39.7% carbohydrate linked to the peptide by N-glycosidic linkages between *N*-acetylglucosamine and asparagine (*4*).

The basic glycoprotein PRP and Ps (parotid size variant-IB8) have been reported to bind buccal cells, bacteria, and dietary tannins. Therefore, a proposed role for the salivary PRPs is as a "first line of defense" against the detrimental effects of polyphenols in the diet (*5*). This binding ability has been previously studied, leading to conflicting results (*6, 7*).

After demonstrating the different behavior of salivary proteins from two individuals in the presence of condensed tannins, we studied the tannin interactions with the various purified salivary proteins. We focused on the glycosylated protein, which was found particular from data of the first

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10.1021/jf801249e CCC: $40.75
Published on Web 09/23/2008

part of this work and is reported to possess lubricating properties (*8, 9*). The objective of this study was to characterize the condensed tannins and the salivary proteins involved in insoluble complexes and "soluble" complexes arising from tannin−salivary protein interactions and to evaluate the effect of the glycosylation of the PRPs.

MATERIALS AND METHODS

Materials. *Reagents.* Solvents and acids (HPLC grade) were purchased from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS) was supplied by Sigma Chemical Co. (Poole, Dorset, U.K.).

Grape (Vitis vinifera Var. Alicante bouchet) Seed Proanthocyanidin Fractions. Grape seeds were ground under liquid nitrogen. The resulting powder was extracted by a mixture of acetone/water (60:40 v/v) and centrifuged (5000*g*, 15 min) to eliminate particle residues. The supernatant was recovered, filtered on GF/C Whatman (Maidstone, Kent, U.K.), and evaporated under vacuum before dissolution in methanol. The proanthocyanidin fraction was obtained by chromatography on a TSK HW-50F column (TosoHaas, Tokyo, Japan) (*10*).

Saliva. Samples were collected between 10:00 a.m. and 12 p.m. to minimize the consequences of diurnal variation observed for salivary constituents (*11*) and after a mouth rinse with water.

P.S.-M. (PSM) is a non wine drinker unable to rate astringency, which she always perceives as very high. J.-M.C.-B. (JMC) is an enologist used to tasting wine and scoring the intensity of astringency. Saliva from these two individuals was independently collected by retaining saliva in the mouth and then expectorating it into an ice-cooled tube. After the addition of EDTA (final concentration of 5 mM), the saliva was centrifuged at 10000*g* for 10 min to remove any insoluble material. The supernatant, referred to as whole saliva, was stored frozen at −20 °C (*12*).

Saliva used for purification was collected from a single individual (JMC).

Determination of Protein Contents. The concentration of proteins was determined by bicinchoninic acid assays using the Pierce BCA assay with bovine serum albumin (BSA) as standard according to the manufacturer's instructions.

Determination of Amino Acid Composition. Protein samples were hydrolyzed in 6 N HCl at 110 °C during 24 h. Amino acids were analyzed on a Biochrom 30 amino acid analyzer (Biochrom, Cambridge, U.K.). Calibration was achieved by comparison with amino acid standards from Sigma Chemical Co.

Electrophoresis. *Gel Running Conditions.* Samples mixed with electrophoresis sample buffer (0.125 M Tris-HCl, 4% SDS; 20% v/v glycerol, 0.2 M DTT, 0.02% bromophenol blue, pH 6.8; v/v) and heated at 100 °C for 5 min were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) following the Laemmli method (*13*) using 12.5% acrylamide separating gels. The stacking gels were 4% acrylamide (Bioprobe, Montreuil-sous-bois, France). Electrophoresis were performed on a Bio-Rad Protean IIxiCell apparatus (Hercules, CA) at 25 mA/gel for stacking gel run and at 35 mA/gel for separating gel run. Apparent molecular weights (M_r) were estimated by comparison with the migration rates of standard proteins (Low Molecular Weight Protein Kit, Amersham Biosciences, Buckinghamshire, U.K.).

Staining Procedure for the Detection of Proteins. The gels were fixed in 100 mL of ethanol/acetic acid/deionized water (40:10:50) for 1 h. The proteins were stained with Coomassie Brillant Blue R250 (CBB, 0.1% in 25% methanol, 10% acetic acid). The CBB destaining step by incubation in 10% acetic acid (methanol or ethanol was omitted) distinguishes PRPs, which stain pink-violet, from other proteins, which stain blue.

Staining Procedure for the Detection of Glycoproteins. A glycoprotein detection kit (Sigma, St. Louis, MO) was used to detect the sugar moieties of glycoproteins on SDS-PAGE. This modifcation of the periodic acid−Schiff (PAS) method yields magenta bands with colorless background. Staining was performed as mentioned by the furnisher, and peroxidase from horseradish, reported as having a carbohydrate content of approximatively 16%, was used as positive control in the kit.

Procedure of Salivary Protein Purification. All operations were performed at 4 °C. Dialyses were done with a 10 kDa molecular weight cutoff membrane.

From whole saliva, proteins were fractionated using a method adapted from that of Oho et al. (*14*). The whole saliva dialyzed overnight against 50 mM potassium phosphate buffer (pH 6.8) was treated with ammonium sulfate to achieve 45% saturation. After stirring overnight, the protein suspension was centrifuged at 20000*g* (Sorvall RC5 Plus centrifuge DuPont, A12.17 rotor Kontron) for 30 min. The supernatant containing proline-rich proteins was dialyzed against 50 mM Tris-HCl buffer (pH 8.6) and applied to a column (1.8 × 11 cm) of DEAE-Sephadex A25 (Pharmacia Biotechnology, Uppsala, Sweden) equilibrated in the same buffer. The column was developed with 42 mL of equilibration buffer, and then bound proteins were eluted with 0.5 M NaCl at a flow rate of 0.3 mL min^{-1}. Two millliliter fractions were collected, and their absorbance was measured at 210, 230, and 280 nm. The nonbound and bound fractions were independently pooled, dialyzed against water, and then lyophilized. The nonbound pooled fractions were dissolved in 10 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$ and applied on a 5 mL affinity column (0.8 × 14 cm) of Con A-Sepharose (Pharmacia Biotechnology), which had been equilibrated with this binding buffer. The column was washed with 25 mL of binding buffer at 0.3 mL min^{-1}, and the bound material was eluted with 10 mM Tris-HCl (pH 7.5) containing 350 mM NaCl and 0.5 M methyl α-D-mannopyranoside (α-MM) at the same flow rate, the flow being held for 15 min during elution. Fractions of 1.8 mL were collected, and absorbance was measured at 210, 230, and 280 nm. The eluted fractions were pooled, dialyzed against water, and lyophilized. This lyophilized sample was dissolved in 50 mM potassium phosphate (pH 6.8) buffer containing 1.7 M ammonium sulfate and applied to a phenyl-Sepharose CL-4B column (2.0 × 11.5 cm) equilibrated in the same buffer for hydrophobic chromatography. After washing the column at a flow rate of 0.5 mL min^{-1} (1 mL fractions collected), the glycosylated PRP was eluted with 50 mM potassium phosphate buffer (pH 6.8) in fractions, which were then pooled, dialyzed, and lyophilized.

Protein purification was monitored by SDS-PAGE according to the method of Laemmli (*13*).

Deglycosylation of Glycosylated PRP (dGPRP). *N*-Glycosidase F can cleave the intact protein without prior denaturation using detergents. The samples were tested for deglycosylation with and without denaturation procedure. The last procedure was chosen for further experiments. N-Linked carbohydrate side chains were released from glycosylated PRP by incubation of the purified protein (20 μg of GPRP) with *N*-glycosidase F (2 units, *N*-glycosidase F, activity = 0.2 unit/μL, specific activity = 25 units/μg of protein, Roche Molecular Biochemicals, Mannheim, Germany) in 0.75 mM ammonium acetate buffer (pH 8) at 37 °C overnight.

Binding Assay. Interaction mixtures (80 μL final volume) contained proteins (around 60 μg of proteins in 40 μL of whole saliva and 20 μg for purified proteins in 40 μL of water) and different amounts of condensed tannins solubilized in 40 μL of 10% ethanol. Binding assays were performed in tubes maintained at 25 °C for 5 min. Then, they were centrifuged for 10 min at 2500*g* at 4 °C. The resulting total mixture (total), supernatant, and pellet were analyzed by thiolysis and SDS-PAGE. Each assay was done in triplicate.

Thiolysis. Proanthocyanidins were characterized and quantified by means of acid-catalyzed depolymerization in the presence of phenylmethanethiol (thiolysis) directly followed by reverse phase HPLC analysis (*15*). The analysis of the released products by thiolysis was performed at 280 nm by external calibration with commercial standard (catechin, epicatechin) and with benzylthioether derivatives purified in our laboratory by semipreparative reverse phase HPLC after thiolysis of grape seed extracts (*10*). The samples (20 μL of supernatant added to 80 μL of methanol or 20 μL of total mixture added to 80 μL of methanol containing 0.2% of SDS or pellets dissolved in 100 μL of methanol containing 0.2% of SDS) were introduced into a glass ampule together with an equal volume of a solution of toluene-α-thiol in methanol containing HCl (0.2 M). After sealing, thiolysis was performed and reaction compounds were separated as described by Rigaud et al. (*15*). The yield of thiolysis degradation was calculated as the ratio

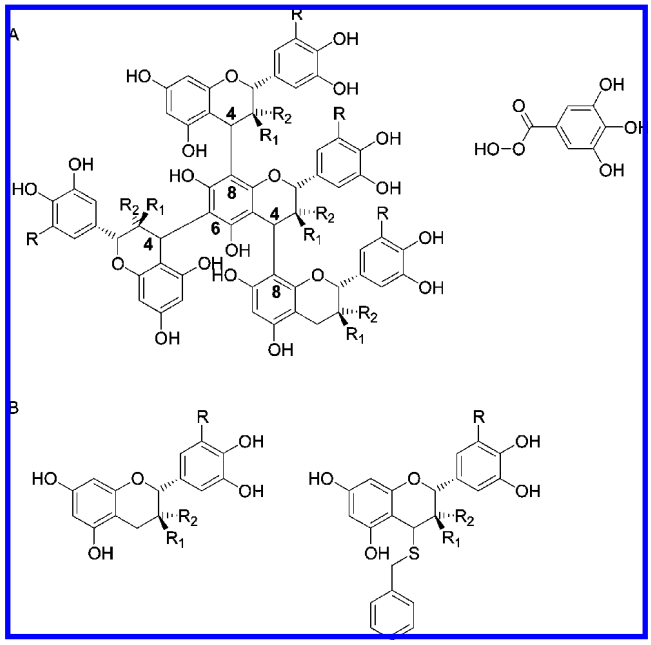

Figure 1. (**A**) Structures of proanthocyanidins; (**B**) monomeric units released after thiolysis (terminal and extension benzylthioether derivatives, X−SR units). R_1 = OH, R_2 = R = H, catechin (C); R_1 = R = H, R_2 = OH, epicatechin (Ec); R_1 = R = H, R_2 = O−G, epicatechin-3-*O*-gallate (EcG); R_1 = H, R_2 = R = OH, epigallocatechin (EgC); R_1 = H, R = OH, R_2 = O−G, epigallocatechin-3-*O*-gallate (EgCG).

between the summed concentrations of the released units (flavan-3-ols and benzylthioethers) and the initial tannin concentration of a control polymeric tannin preparation and estimated for each experiment (from 72 to 85%). All experiments were done in triplicate. One analysis was performed per sample.

Data Analysis. Data treatments were performed using the Matlab software (version 7.3, The Mathworks Inc., Natick, MA).

RESULTS AND DISCUSSION

Tannin Characteristics. Tannins (**Figure 1A**) are characterized by the nature of their extension and terminal flavan-3-ol units. The principle of thiolysis is as follows: polymeric proanthocyanidins are cleaved into monomeric units, which are released as flavan-3-ol if they are terminal units and as benzyl thioethers (**Figure 1B**) if they are upper and extension units (*16*). Thus, it allows structural analysis [mean degree of polymerization (mDP), percent of EgC, percent of galloylated units] of tannins. This is of primary importance because many works have reported that interactions between tannins and proteins (*17*−*20*) and the perception of astringency (*21*−*23*) depend on tannin structure.

The mDP of the tannins determined by thiolysis was 6.84, and the average proportion of galloylated units was 20.57%, in agreement with our previous data (*10*).

Electrophoretic Pattern of Saliva. SDS-PAGE is used to analyze human salivary proteins (*24, 25*). **Figure 2A** shows the SDS-PAGE of whole saliva. Proteins are the only components detected on the gel. The Coomassie Blue (CBB R-250) staining is directly related to the amount of proteins in the mixture. PRPs are characterized by their particular metachromatic staining with Coomassie Brillant Blue R-250 (*24*). As they migrate abnormally slowly on gel electrophoresis and because there are no PRPs that can be used as markers (*26*), the molecular weight (M_r) given is an apparent M_r. Migration of proteins in SDS-PAGE is affected by the extent of glycosylation; thus, glycoproteins can broaden a single protein band into a smear on gels

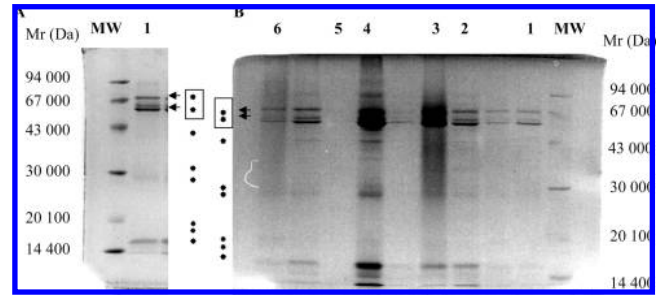

Figure 2. (**A**) SDS-PAGE of whole saliva; (**B**) SDS-PAGE of (1) PSM whole saliva (load protein = 8.35 μg), (2) JMC whole saliva (load protein = 6.66 μg), (3) pellets obtained after binding tannins (25 μg) with PSM saliva (protein load if total precipitation = 52 μg), (4) pellets obtained after binding tannins (25 μg) with JMC saliva (protein load if total precipitation = 66 μg), (5) PSM corresponding supernatant (loaded volume of supernatant = 10 μL), and (6) JMC corresponding supernatant (loaded volume of supernatant = 10 μL). ∗ indicates PRPs, and arrows indicate α-amylase. Scarred area shows the PAS reactive PRPs.

(*27*). In **Figure 2A**, lane 1, the whole saliva protein profile shows a large number of PRP pink-violet bands with M_r between 97 and 13 kDa. The broad, pink-staining upper band was identified as the major glycosylated PRP, a PAS-positive protein band with a M_r between 97 and 66 kDa. Intensely blue-stained bands were detected around 51 kDa. They correspond to α-amylase and its glycosylated isoform. Pink-staining bands around 21−14 kDa and extremely sensitive to the staining/destaining procedure are basic PRPs (IB1−IB9).

The estimation of proteins by BCA procedure showed that the JMC saliva (**Figure 2B**, lane 2) and the PSM saliva (**Figure 2B**, lane 1) concentrations, 1.67 and 1.3 mg mL^{-1}, respectively, and compositions are comparable. However, the JMC saliva showed a more pink-stained profile than PSM saliva, particularly in the 90−57 kDa area corresponding to the glycosylated PRP.

Tannin−Saliva Interactions. As previously observed, mixing whole saliva and condensed tannins (*28*) gives rise to a "soft cloudy" precipitate, which gathered after centrifugation on the bottom of the tube so that the supernatant was easily recovered without disturbing this pellet. No precipitate was formed in the control tannin solution.

The tannin composition was determined by HPLC after thiolysis. Nine variables related to tannin composition were taken into account: amounts of tannins in the total (a*T*), pellet (a*P*), and supernatant (a*S*), mDP in total (DP*T*), pellet (DP*P*), and supernatant (DP*S*), and the percent of galloylated tannin units in the total (gal*T*), pellet (gal*P*), and supernatant (gal*S*).

In former experiments (*19, 29*), SDS treatment was shown to increase recovery of tannins from both insoluble tannin−protein complexes and supernatants containing soluble complexes. The amounts of tannins in the supernatant and in the pellet account for the initial amount added to the binding mixture.

SDS treatment was performed on pellets and totals but not on supernatants. Around 30 and 85% of the tannins introduced in the solution are precipitated by JMC saliva and PSM saliva, respectively (**Table 1**). For JMC saliva, 30% of the tannins present in the original sample were recovered neither in the pellet nor in the supernatant. They were partly (20%) recovered from the total after treatment with SDS, meaning that they were in the supernatant. As the supernatant was not SDS-treated, the "loss" of tannins in JMC samples was due to tannin trapping in soluble complexes in the supernatant. The percentage of tannins in these soluble complexes was almost unchanged for the

Table 1. Tannin Amounts Obtained after Binding with Whole Saliva from Individuals JMC and PSM in Pellet (a*P*), Supernatant (a*S*), and Total Mixture (a*T*), Percentage of Precipitated Tannins, and Percentage of Unrecovered Tannins in a*S* + a*P* and in a*T* with Regard to Theoretical Initial Amount (a*I*)

	JMC Saliva			
a*I* (μg)	6.25	12.5	18.5	25
a*P* (μg)	1.55 ± 0.3	3.86 ± 0.07	6.31 ± 0.8	8.89 ± 0.5
a*S* (μg)	2.04 ± 0.09	4.42 ± 0.11	6.76 ± 0.28	9.36 ± 097
a*T* (μg)	8 ± 1.0	11 ± 0.37	16.6 ± 0.81	22.9 ± 0.81
a*T* − (a*P*+ a*S*)		2.72	3.53	4.65
precipitated tannins %	24.8	30.8	34.1	35.6
loss % [a*I* − (a*P* + a*S*)/a*I*]	27	34	30	27
loss % (1 − a*T*/a*I*)	0	12	11.5	8.4

	PSM Saliva			
a*I* (μg)	6.25	12.5	18.5	25
a*P* (μg)	5.3 ± 0.5	11.7 ± 0.24	15.66 ± 1.5	20.52 ± 0.8
a*S* (μg)	0.65 ± 0.04	1.4 ± 0.23	2.35 ± 0.43	4.31 ± 0.31
a*T* (μg)	5.4 ± 0.44	12.3 ± 1.0	18.6 ± 0.9	27 ± 4.0
a*T* − (a*P* + a*S*)	0	0	0.1	2.2
precipitated tannins %	84.8	93.6	84.6	82
loss % [a*I* − (a*P*+ a*S*)/ a*I*]	4.8	0	3.9	1
loss % (1 − a*T*/a*I*)	13.6	2	1	0

Table 2. Analysis of Covariance (*F* Value), Results for the Nine Variables Measured (See Text for Variable Labels)[a]

	F value		
	tannin amount	nature of saliva	amount × saliva
a*T*	267.5[a]	0.5	13.3[a]
a*P*	501.9[a]	485.7[a]	57.7[a]
a*S*	492.1[a]	359.3[a]	57.2[a]
DP*T*	1.4	1.5	0.0
DP*P*	39.3[a]	113.7[a]	4.2
DP*S*	3.1	0.5	0.0
gal*T*	8.5[a]	4.2	0.5
gal*P*	0.8	6.0[a]	2.3
gal*S*	0.4	1.1	0.2

[a] Significant effect at a probability level of 5%.

different tannin amounts tested. The PSM saliva was unable to form soluble complexes with tannins.

To investigate the effects of added tannin amounts (four levels) and the nature of saliva (two levels: JMC/PSM), a covariance model analysis was fitted for each of the nine variables. This covariance model takes into account the interaction term between these two effects by comparison of the linear regression calculated for each saliva (**Table 2**). The equality of slopes obtained for each nature of saliva after the linear regression (variable) = *f*(amount) was tested considering the interaction term between these two factors. Added tannin significantly (at a probability level of 5%, **Table 2**: footnote *a*), affected the contents in all compartments, as expected, and the mDP in the pellet, meaning that the selectivity of precipitation was dose dependent. For a*P*, a*S*, DP*P*, and gal*P*, the factor "nature of saliva" was significant. The effect of the interaction term between saliva and amount was significant on a*T*, a*P*, a*S*,, and DP*P*. Dose responses were different for both salivas, as expression of a*P* and a*S* as linear regressions of initial amount (a*I*) a*P* = f(a*I*) and a*S* = f(a*I*) showed different slopes.

Higher mDP tannins were selectively precipitated, as shown earlier (*20, 28*). Moreover, large differences between salivas were observed; tannins were involved in insoluble complexes showing mDP values of 7.8 and 10 with JMC and PSM salivas, respectively.

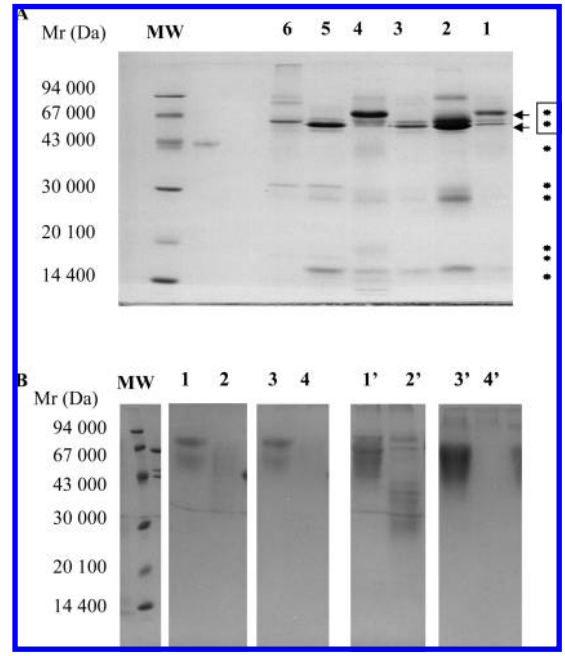

Figure 3. (**A**) SDS-PAGE of the salivary protein purification. Lanes: (1) ammonium sulfate 45% supernatant; (2) ammonium sulfate 45% pellet; (3) DEAE nonretained fraction; (4) DEAE eluted fraction; (5) Con-A nonbinding proteins; (6) Con-A binding proteins. (**B**) *N*-Glycosidase F digestion of GlycoPRP obtained after hydrophobicity chromatography. Lanes 1, 3, 1′, and 3′ are GlycoPRP only; lanes 2, 4, 2′, and 4′ have been digested with *N*-glycosidase F. Lanes 1, 2, 1′, and 2′ have been Coomassie Blue stained. Lanes 3, 4, 3′, and 4′ were stained by periodic acid−Schiff method (PAS). ∗ indicates PRPs, and the arrows indicate α-amylase. Scarred area shows the PAS reactive PRPs.

The amounts of protein introduced in JMC and PSM interaction mixtures are comparable; therefore, the amounts of proteins were not the limiting factor driving the formation of soluble complexes that can be involved in astringency. The protein patterns of the pellets (**Figure 2B**, lanes 3 and 4) were qualitatively comparable. The basic PRPs were particularly precipitated, the JMC pellet (around 30 μg of protein) showing more defined bands than PSM (52 μg). Supernatant analysis showed no proteins in the PSM supernatant (lane 5). On the contrary, the JMC supernatant (lane 6) showed remaining proteins (30 μg), which may be involved in soluble complexes still suggested by data obtained on nonrecovered tannins. Besides, the glycosylated PRP appeared to be particularly abundant in the JMC supernatant.

Statistical analysis showed that the behaviors of JMC and PSM salivary proteins are discriminated by amounts of tannins both in the supernatant and in the pellet and by their percent of galloylation (gal*P* = 23 and 20.5% for JMC and PSM salivas, respectively). JMC produces proteins that are involved in soluble complexes and require higher levels of tannins to be precipitated. In that case, the lubrication would be maintained longer. The preferential interaction with high mDP galloylated tannins, which are the most astringent (*30*), and the presence of protein−tannin complexes involving glycosylated PRP strongly suggest the importance of glycosylation for interaction with tannins and in the formation of soluble complexes. To evaluate this feature, we developed interaction studies involving human purified saliva proteins.

Purification of Salivary Proteins. Figure 3 shows the SDS-PAGE electrophoregram of the purification steps used to obtain the different classes of salivary proteins. The Coomassie Brillant Blue R-250 staining and PAS staining were applied in parallel.

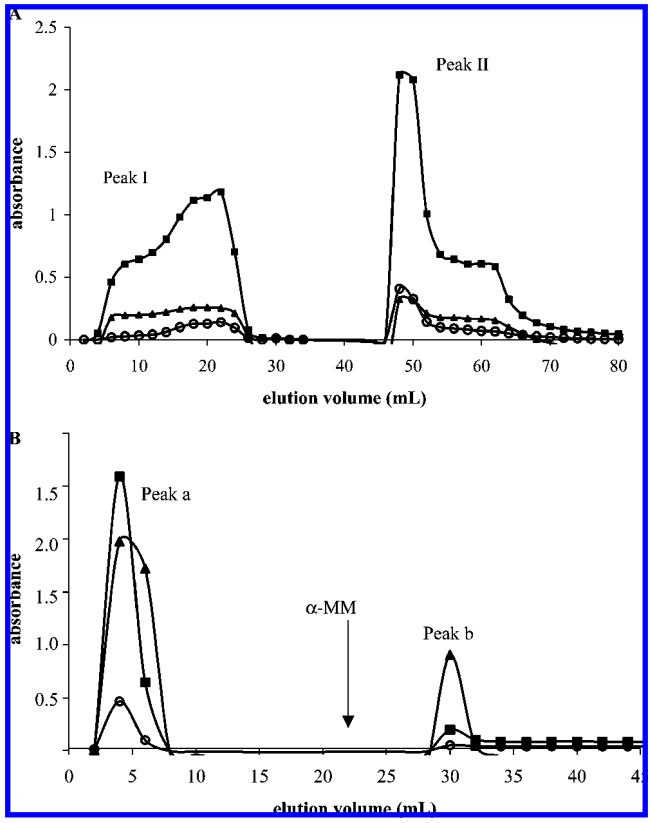

Figure 4. Purification of salivary proteins. (**A**) Ion-exchange chromatography. The dialyzed ammonium supernatant was passed through DEAE-Sephadex equilibrated with Tris-HCl buffer (50 mM, pH 8.6) washed with 42 mL of the same buffer and eluted with 0.5 M NaCl. (**B**) Affinity chromatography on Con A−Sepharose (0.8 × 14 cm). After sample application, the column was washed with 10 mM Tris-HCl buffer (pH 7.5) containing containing 150 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$. The bound material was eluted with 10 mM Tris-HCl (pH 7.5) containing 350 mM NaCl and 0.5 M methyl α-D-mannopyranoside (α-MM) as indicated by the arrow. Sample fractions were monitored by their absorbance at λ: ▲, 210 nm; ■, 230 nm; ○, 280 nm.

The dialyzed whole saliva was submitted to an ammonium sulfate procedure that allowed the elimination of a part of the acidic PRPs and of α-amylase (**Figure 3A**, lane 2). Basic PRPs were not precipitated by ammonium sulfate 45% and were recovered in the supernatant (**Figure 3A**, lane 1) together with some acidic PRPs. This supernatant containing 24 mg of proteins was applied on a column of DEAE-Sephadex A-25, a weak anion type exchanger. The elution profile of the DEAE-Sephadex chromatography (**Figure 4A**) showed basic and glycosylated PRPs excluded from the column with 42 mL of the starting buffer (4 mg of proteins; **Figure 4A**, peak I; **Figure 3A**, lane 3); then, applying 0.5 M NaCl allowed the elution of acidic PRPs (6.6 mg of proteins; **Figure 4A**, peak II; **Figure 3A**, lane 4). The DEAE nonretained proteins were submitted to Con-A affinity chromatography. From the extensive wash of the column with the binding buffer, unbound proteins were excluded (**Figure 4B**, peak a; **Figure 3A**, lane 5), whereas the bound material (**Figure 4B**, peak b; **Figure 3A**, lane 6) was eluted with α-MM. The SDS-PAGE analysis of this Con A+ fraction stained with Coomassie Brillant Blue showed two bands and a few minor bands. The major one corresponds to 69−55 kDa broadband and the other one to 56 kDa. Staining with periodic acid−Schiff reagent (PAS) allowed the detection of the broadband at 69 kDa, confirming its glycosylated state. The Con A+ fraction obtained from this procedure contained predominantly the glycosylated PRP (GPRP) and minor contaminants of α-amylase as previously observed by Oho et al. (*14*). Hydrophobic chromatography used as the final purification step allowed to obtain GPRP. The SDS-PAGE profile showed a band stained with the Coomassie Brillant Blue (**Figure 3B**, lanes 1 and 1′) and PAS reagent (**Figure 3B**, lanes 3 and 3′). Treatment with PNGase of this fraction gave a protein (**Figure 3B**, lanes 2 and 2′) that did not stain with periodate−Schiff regeant any more (**Figure 3B**, lanes 4 and 4′), suggesting that all of the oligosaccharides N-linked to the protein backbone were eliminated. Upon deglycosylation, the product showed a major band with an apparent M_r of 35000 Da, consistent with previous data (*7, 26*); the presence of more than one polypeptide chain in the deglycosylated PRP may be due to the copurification of different gene products or postribosomal cleavage of the primary translation product (*7*). Amino acid analysis confirmed their PRP nature and was in agreement with published data. From 66 mL of JMC saliva containing 100 mg of proteins, 1 mg of PRPs and 360 μg of glycosylated PRP were purified.

Table 3. Tannin Content Obtained in Pellet after Binding with PRP, GlycoPRP, and dGPRP, Percentage of Precipitated Tannins with Regard to Theoretical Initial Amount, and Average DP (mDP) of Precipitated Tannins and Recovered in the Supernatant[a]

initial tannin amount		PRP	GlycoPRP	dGPRP
6.25 μg	precipitated tannin amount (μg)	3.39 ± 0.27	2.19 ± 0.27	5.06 ± 0.38
	% of precipitation	65.26	34.06 ± 1.76	80.99 ± 6.0
	mDP of precipitated tannin	6.37 ± 0.69a	5.09 ± 0.55c	10.53 ± 0.47e
	mDP in the supernatant	2.58 ± 1.17b	3.75 ± 0.01d	1.68 ± 0.15
25 μg	precipitated tannin amount (μg)	18.55 ± 0.19	17.74 ± 1.16	25.37 ± 0.47
	% of precipitation	93.5 ± 11	70.97 ± 4.66	100
	mDP of precipitated tannin	7.12 ± 0.4a	6.84 ± 0.45a	6.32 ± 0.21a
	mDP in the supernatant	2.73 ± 0.65b	4.33 ± 0.12d	

[a] Values with different letters differ ($p < 0.05$).

Tannin−Protein Interactions. Using a competitive binding assay between BSA and a 158 kDa submandibular PRG from rat, Asquith et al. (*6*) have demonstrated a higher affinity of this latter protein for tannins and suggested the presence of soluble complexes without precipitation. However, we demonstrated from saliva that human glycosylated PRPs, involved in the lubricating ability of saliva (*9*), may establish interactions with polyphenols leading to soluble polyphenol−protein associations, which could afterward precipitate. To evaluate and compare the ability of these proteins to interact with tannins and in particular the influence of glycosylation, we have examined the effect of condensed tannins on purified PRPs, glycosylated PRP, and its deglycosylated form (dGPRP). The available amounts of purified proteins have limited the tannin amount studies. Therefore, 20 μg of purified protein was introduced in the interaction mixtures together with 6.25 or 25 μg of proanthocyanidins.

The abilities of grape seed tannins to precipitate with PRPs, GlycoPRP, and dGPRP have been quantitatively and qualitatively studied (**Table 3**).

Precipitation of tannins with PRPs (65%) exceeded that observed with JMC whole saliva (30%), meaning that PRPs alone are more efficient than a mixture of proteins. As previously noted with individual saliva, the level of precipitation is related to the nature of the proteins. These data were in accordance

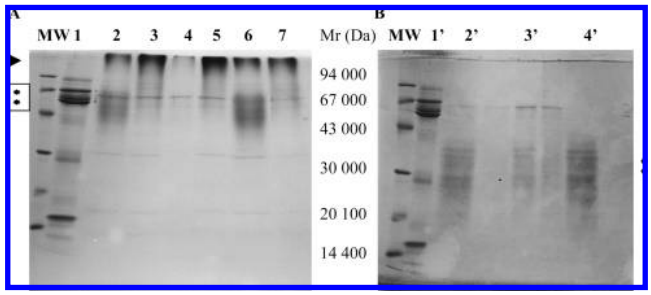

Figure 5. Glycosylated PRP (**A**) and deglycosylated PRP (**B**) binding to tannins. Lanes 1 and 1′ are whole saliva controls; lanes 2 and 2′ are total interaction mixture with 5 μg of tannins; lane 3 is total mixture with 25 μg of tannins; lanes 4 and 4′ are pellet obtained with 5 μg of tannins and lane 5 is that obtained with 25 μg; lanes 6, 3′, and 7 show the corresponding supernatants. ∗ indicates PRPs, square area shows the PAS reactive PRPs, and the arrowhead indicates tannin−glycosylated PRP complexes.

with nephelometric measurements (*31*), which showed that saliva fraction with α-amylase had lower affinities for polyphenols than the global PRP fraction used by the authors. In the presence of PRPs and at low tannin concentrations, the tannins recovered in the pellets were not significantly different from those of the initial mixture and oligomers remained in solution meaning that, at this tannin−protein ratio, the precipitation affected all proanthocyanidins except the lowest molecular weight molecules. When the amount of added tannins was increased (25 μg of tannins), precipitation of the highest molecular weight proanthocyanidins was observed. The increase of mDP in the supernatant suggested that the precipitation of the largest molecular weight tannins protects oligomers from precipitation. The precipitation of large proanthocyanidins, whereas the dimeric and trimeric structures remain in the supernatant, suggests that the affinity of proanthocyanidins for salivary proline-rich proteins is related to the number of phenolic rings as has been demonstrated for galloyl esters by several methods including precipitation of hemoglobin from aqueous solution (*32*). Recently, the use of microcalorimetry experiments (*33*) has shown that the largest and most hydrophobic polyphenols bind primarily to poly(L-proline) and that the binding interactions may arise from entropic contributions that are correlated with conformational changes.

Interestingly, in the presence of glycosylated PRP and at low concentration of tannins the amount of precipitation was largely reduced. The deglycosylation of GPRP allowed the recovery of a precipitation level comparable to the one obtained for PRPs. Electrophoresis patterns (**Figure 5A**) showed the presence of a particular product which did not enter the gel that corresponds to GPRP−tannin complexes. At low tannin concentration, the total mixture (lane 2) and the supernatant (lane 6) contained the glycoPRP and its tannin-bound form. At high concentration of tannins, the presence of the tannin-bound form was detected in all of the compartments (lanes 3, 5, and 7) and no free glycosylated PRP was detected. The corresponding experiments performed with the deglycosylated PRP (**Figure 5B**) showed no tannin-bound form on top of the gel. These results confirmed that carbohydrate moieties prevent the precipitation of the complexes which are formed. If protein content is held constant and polyphenol concentration is increased, the precipitation increased as observed for haze by Siebert (*34*). The interaction with tannins reduced the apparent stabilizing effect of glycosylation. From the literature, the aggregation-regulating effect of glycosylation has been directly related to the kinetic partitioning between folding and aggregation, and this may provide a potential explanation for the glycoPRP behavior in the presence of tannins. At low concentration of tannins, the precipitation could be inhibited by the reduced hydrophobicity as a result of glycosylation. At high tannin concentration, it may be enhanced by the increased hydrophobicity as a result of increased binding of tannins.

Recently the human salivary basic PRP, IB5, has been clearly confirmed as an intrinsically unstructured proteins (IUPs or IDPs for intrinsically unstructured or disordered proteins) able to develop a binding-coupled folding process in the presence of polyphenols (*35*). A partial structuring of the C terminus of neurotensin, a linear tridecapeptide in the presence of polyphenols, has been also described (*36*). The glycosylated protein may act in a similar way. Protein−tannin interactions were approached by using models between salivary proteins and grape seed condensed tannins. The extent of interaction was estimated by assaying tannins in the insoluble complexes and also in the soluble ones. Proteins were studied by means of electrophoresis and condensed tannins by thiolysis. We have demonstrated different abilities of salivary proteins purified from human saliva to bind condensed tannins. The results on individual saliva showed that the different protein patterns led to different tannin binding. The link to a different propensity to astringency perception is still under question. It was found from purified proteins that the glycosylation of human PRPs favors the formation of soluble complexes and reduces tannin precipitation with regard to tannin amounts. The presence of polysaccharide moieties on PRPs allows the formation of soluble complexes that may be able to preserve lubrication in the mouth. Increasing the amounts of tannins leads to larger precipitation of proteins, in particular that of glycosylated proline-rich proteins. In that way, the presence of a large amount of tannins in a wine sensed as astringent, corresponding to a low salivary protein−tannin ratio in the mouth, could favor the precipitation of the glycosylated PRPs the presence of which in the saliva in a high proportion might delay astringency. The interaction with tannins is thought to reduce significantly the lubricating qualities of human saliva both by decreasing its viscosity and by increasing friction, both factors lending support to the notion that astringency is a tactile phenomenon. The preferential interaction with higher mDP tannins, which are the most astringent ones (*30*), and the presence of protein−tannin complexes in the JMC saliva further support this hypothesis.

From a biological point of view, an organism may take advantage of owning a series of tannin-binding proteins available to ensure the effective complexation of the various tannins that occur in food. These polymorphic variations could form the basis of differences in oral microflora and susceptibility to oral infections (*37*) as tannins would compete for PRPs. Cross-cultural preferences for tannin-rich beverages such as tea, coffee, and red wine may be explained by reduction in adhesion of food particles to the oral mucosa, allowing their rapid oral clearance and modulating astringency perception. Our results taken together with previous findings suggest that the multifunctional nature of these salivary proteins helps to explain both the subtle and large variations found in structure, secretion of proteins, and perception between individuals. Future studies will explore the variability in salivary protein profile of a children's panel to examine if there is some criteria to distinguish individuals whose saliva is more or less able to bind polyphenols and consequently "protected" and eventually sensible to astringency.

From a molecular point of view, studies to discover the origins of the observed differences in interaction rates have to be examined thoroughly. The nature of the salivary PRPs as IUPs and their characteristic properties of binding tannins mean that one must take a multidisciplinary approach to study them. NMR, mass spectrometry (MS), and circular dichroism (CD) are used to study protein

folding or solution conformations, but they have to be adapted as a tool for studying IUPs and therefore tannin−protein interactions. Our further objective is to develop these approaches with two human salivary proteins that have been produced by heterologeous expression (*38*) and consequently to achieve better understanding of the mechanisms responsible for the complexation of tannins, which may thereby minimize their detrimental biological effects and may influence astringency.

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Received for review April 21, 2008. Revised manuscript received July 28, 2008. Accepted August 2, 2008.

JF801249E