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Reactivity of Human Salivary Proteins Families Toward Food Polyphenols

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ABSTRACT: Tannins are well-known food polyphenols that interact with proteins, namely, salivary proteins. This interaction is an important factor in relation to their bioavailability and is considered the basis of several important properties of tannins, namely, the development of astringency. It has been generally accepted that astringency is due to the tannin-induced complexation and/or precipitation of salivary proline-rich proteins (PRPs) in the oral cavity. However, this complexation is thought to provide protection against dietary tannins. Neverthless, there is no concrete evidence and agreement about which PRP families (acidic, basic, and glycosylated) are responsible for the interaction with condensed tannins. In the present work, human saliva was isolated, and the proteins existing in saliva were characterized by chromatographic and proteomic approaches (HPLC-DAD, ESI-MS, sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), and MALDI-TOF). These approaches were also adapted to study the affinity of the different families of salivary proteins to condensed tannins by the interaction of saliva with grape seed procyanidins. The results obtained when all the main families of salivary proteins are present in a competitive assay, like in the oral cavity, demonstrate that condensed tannins interact first with acidic PRPs and statherin and thereafter with histatins, glycosylated PRPs, and bPRPs.

KEYWORDS: Astringency, condensed tannins, proline-rich proteins, salivary proteins

INTRODUCTION

Tannins are polyphenols commonly found in plant-derived foods. Tannins are classically divided into two major classes: condensed tannins (proanthocyanidins), which are polymers of catechin, and hydrolyzable tannins, which are gallic or ellagic esters of glucose. Although tannins have benefits for human health regarding their antioxidant properties,[1−3] they can have a number of harmful effects including decrease in growth and body weight gain,[4] and inhibition of digestive enzymes.[5] Regarding their antioxidant properties, an in vitro study[1] showed that independently of the degree of polymerization, condensed tannins provide protection of lipidic peroxidation, but this effect is increased for the less complex structures. The same behavior was shown for condensed tannins against tumor cell viability and proliferation.

One of the characteristics of tannins is their ability to precipitate certain proteins, and this ability has been considered the basis of the sensation of astringency on the human palate. Astringency has been defined as a complex group of sensations involving dryness and tightening of the oral surface and puckering sensations of the oral cavity.[6,7] It was proposed by Bate-Smith[7,8] that astringency results from the interaction of tannins with salivary proteins (SP) in the mouth, and since then, it has been generally accepted and supported by the literature[9−12] that astringency is due to the tannin-induced interaction and/or precipitation of salivary proline-rich proteins (PRPs) in the oral cavity. However, astringency is a very complex sensory experience, and the possible mechanisms for its development are presently controversially discussed in the scientific community.[13] Some studies point to the importance of the interaction of salivary glycoproteins with tannins with consequent modifications of their viscous properties and oral cavity delubrication,[14,15] and also, the salivary flow rate appears to be correlated with astringency intensity.[16] The involvement of the cells of the oral cavity in the development of astringency has also been suggested.[17]

Whole saliva represents a mixture of the secretions of the major (submandibular, sublingual,and parotid) and minor salivary glands, together with the crevicular fluid, bacteria, and cellular debris. The secretions from the different glands have been shown to differ considerably and to be affected by different forms of stimulation, day time, diet, age, gender, several disease states, and pharmacological agents.[18]

In general, saliva is composed of proteins, electrolytes, and small organic compounds. With respect to the proteinaceous component, saliva similarly to other bodily fluids, presents a wide range of small molecular weight components that are assigned by several authors as salivary peptides, all species with a m/z below 20 kDa. Salivary peptides have been grouped into six

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structurally[19] related major classes, namely, histatins, basic proline-rich proteins (bPRPs), acidic proline-rich proteins (aPRPs), glycosylated proline-rich proteins (gPRPs), statherin, and cystatins. These peptides have important biological functions in saliva associated with calcium binding to enamel, maintenance of ionic calcium concentration (PRPs and statherin), associated with antimicrobial action (histatins and cystatins), or protection of oral tissues against degradation by proteolytic activity such as cystatins.[20−28]

There is a lot of detailed information for all these families of SP. The family of PRPs is divided into three classes: acidic, basic, and glycosylated. More than 11 human basic-PRPs and five acidic PRP isoforms have been identified.[29,30] Several closely related acidic proline-rich phosphoproteins (aPRPs) were identified by isolation (proteins A and C,[31] PRP1, 2, 3, and 4[32]) or by studies of protein polymorphism (identified as PIF-s, PIF-f, Db-s, Db-f, and Pa). Although the nomenclature of this family of proteins is somehow confusing, in general, all these proteins are isoforms.[33] In general, the designations PRP1, 2, 3, and 4 will be used here including the following alternative names: PRP1/PRP2-PIF-s, Pa, and Db-s; PRP3/PRP4-PIF-f and Db-f. Histatins are a family of small, histidine-rich proteins secreted by the parotid and submandibular glands.[21] Statherin is secreted by the parotid gland and is abundant in tyrosine residues.[20] Cystatins are natural inhibitors of cysteine proteinases.[34]

In the past years, several research groups studied the molecular basis of the development of astringency using models bioassays with pure/isolated PRPs (or similar proteins) and tannins.[35−43] However, only few works had used whole saliva to study the onset of astringency.[44,45]

Several studies have been carried out to evaluate interactions between polyphenols and SP using different techniques, namely, SDS−PAGE,[35,42,46] spectrophotometry,[37,43] nephelometry,[38,40,47] NMR,[36,48] DLS (dynamic light scattering),[15,49] and mass spectrometry.[50] Despite the several techniques applied, there are some difficulties in correlating the perceived astringency to a single physical−chemical phenomenon. To our knowledge, there are no experimental evidence about the relative affinity of different PRP families (acidic, basic, and glycosylated) individually or in a competitive/associative medium such as whole saliva. In fact, the first studies that analyzed salivary protein (SP) interaction with polyphenols by HPLC were done by Kallithraka and co-workers.[44,51] They analyzed human saliva before and after the interaction with polyphenols ((+)-catechin, (−)-epicatechin, procyanidin B2, or procyanidin C1) by HPLC. However, the SP involved in the interaction were not identified.

In the present work, chromatographic and proteomic approaches (HPLC-DAD, ESI-MS, sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), and MALDI-TOF-MS) were developed in order to study the affinity of different families of SP by the interaction of saliva with grape seed procyanidins.

MATERIALS AND METHODS

Materials. All reagents used were of analytical grade or better. Hydrochloric acid and acetonitrile were purchased from Panreac Quimica, acetic acid was purchased from Carlo Erba Reagents, sodium acetate and trifluoracetic acid (TFA) were purchased from Fluka Biochemica, and ethanol was purchased from AGA, Álcool e Géneros Alimentares, SA. Trizma base, glycerol, sodium dodecyl sulfate, β-mercaptoethanol, acrylamide, N',N'-methylenebisacrylamide, tricine, tetramethylethylenediamine, ammonium persulfate, ammonium bicarbonate, formic acid, SigmaMarker Wide Range, molecular weight 6.5−200.0 kDa, and sodium thiosulfate were purchased from Sigma. Bromophenol blue, periodic acid, Schiff's reagent, and Fuchsin-sulfite reagent were purchased from Sigma-Aldrich. Imperial Protein Stain was purchased from Thermo Scientific (United Kingdom). Potassium metabissulfite was from BDH Chemicals Ltd. Sequencing-grade modified trypsin (porcine) was from Promega (Portugal). α-Cyano-4-hydroxycinnamic acid was purchased from Applied Biosystems (Germany). Silver nitrate, sodium carbonate, and formaldehyde were purchased from Merck (Germany).

Grape Seed Fraction (GSF) Isolation. Condensed tannins were extracted from *Vitis vinifera* grape seed extract. This extract was fractionated through a TSK Toyopearl HW-40(s) gel column (100 mm × 10 mmi.d., with 0.8 mL·min^{-1} methanol as eluent), yielding two fractions according to the method described in the literature.[52]

The first fraction was obtained after elution with 99.8% (v/v) methanol during 5 h (240 mL) and the second one after elution with methanol/5% (v/v) acetic acid during the next 14 h (670 mL). Both fractions were mixed with deionized water, and the solvent was eliminated using a rotary evaporator under reduced pressure at 30 °C and then freeze-dried. The procyanidin composition of fractions was determined by direct analysis by ESI-MS (Finnigan DECA XP PLUS).

The first fraction contains mainly catechins (m/z = 290), procyanidin dimers, and their galloyl derivatives, and the second fraction contains procyanidin dimers galloylated, procyanidin trimers, and their galloyl derivatives and procyanidin tetramers. The latter has a mean MW of 936 and a polymerization degree average of 3.2. Only the second fraction named grape seed fraction (GSF) was used because it is composed of more polymerized and galloylated procyanidins, which are known to be more reactive toward proteins.[38,53]

Saliva Collection, Treatment, and Analysis. Whole human saliva was always collected freshly at 2 pm from a healthy, nonsmoking female volunteer. The saliva samples were taken under unstimulated conditions and after at least 1 h without ingestion of food or beverages. Collection time was standardized in order to reduce concentration variability connected to circadian rhythms of secretion.[30] As the objective of this work was to establish a relationship between different families of PRPs (basic, acidic, and glycosylated PRPs) saliva was supplied only by one volunteer in order to simplify the proteomic analysis and chromatogram division.

TFA solution (10% aqueous TFA) was immediately added to 900 μL of collected saliva (1:90 v/v), and the solution was centrifuged at 8000g for 5 min. After centrifugation, the supernatant (acidic saliva, AS) was separated from the precipitate, and 90 μL was immediately analyzed on a HPLC-DAD Elite Lachrom system (L-2130) equipped with a Vydac C8 column, with 5 μm particle diameter (column dimensions 150 × 2.1 mm); detection was carried out at 214 to 280 nm, using a diode array detector (L-2455). The HPLC solvents were (eluent A) 0.2% aqueous TFA and (eluent B) 0.2% TFA in ACN/water 80/20 (v/v). The gradient applied was linear from 10 to 40% (eluent B) in 60 min, at a flow rate of 0.30 mL/min. After this program, the column was washed with 100% eluent B for 20 min in order to elute S-type cystatins and other late-eluting proteins. After washing, the column was stabilized in the initial conditions.

Twelve selected salivary fractions containing different families of salivary proteins were obtained during the HPLC analysis by separate collection of the eluent deriving from the diode array detector.

SDS−PAGE. The 12 selected salivary fractions were dried in a SpeedVac and dissolved in 25 μL of 1× electrophoresis sample buffer (50 mM Tris-HCl, pH 6.8, 12% v/v glycerol, 4% SDS, 2.5% v/v β-mercaptoethanol, and 0.01% bromophenol blue) and heated at 60 °C for 1 h with shaking. The 12 samples were analyzed by SDS−PAGE in a

tris-tricine buffer system according to the method of Schägger[54] using 16% acrylamide resolving gel. The stacking gel was 5% acrilamide. The cathode buffer was 0.1 M Tris, 0.1 M tricine, and 0.1% SDS, and the anode buffer was 0.2 M Tris-HCl, pH 8.9. Electrophoresis was performed on a Bio-Rad MiniProtean Cell electrophoresis apparatus (Bio-Rad). After electrophoresis, the gels were stained with Imperial Protein Stain, a Coomassie R-250 dye-based reagent, or silver stained. The staining with Imperial Protein Stain was done according to the supplier's instructions. The destaining step was done by washing the gels with water until the bands were visible. Molecular weights were estimated by comparison with the migration rates of standard proteins. The silver staining procedure was done according to O'Connell and Stults.[55]

Periodic Acid Schiff's (PAS) Staining. The PAS staining was done according to Zacharius.[56] After electrophoresis, the gels were fixed overnight (40% ethanol and 7% acetic acid) and then immersed in a solution of 1% periodic acid for 60 min. The gels were washed with water and incubated in the dark with Schiff's reagent for 60 min. The gels were then washed three times (0.58% potassium metabissulfite and 3% acetic acid).

Tryptic Digestion. The protocol used for tryptic digestion was according to Vitorino et al.[57] After electrophoresis, the bands of interest were excised from the gel and transferred to a rack. The gel pieces were washed twice with 25 mM ammonium bicarbonate/50% ACN, one time with 100% ACN, and after the washes, the gel pieces were dried in a SpeedVac (Thermo Savant). Twenty microliters of 10 μg/mL trypsin in 50 mM ammonium bicarbonate was added to the dried residue, and the samples were incubated overnight at 37 °C.

After the incubation, the extraction of tryptic peptides was performed by the addition of 10% formic acid/50% ACN three times, followed by lyophylization in a SpeedVac (Thermo Savant). Tryptic peptides were resuspended in 10 μL of a 50% ACN/0.1% formic acid solution.

Mass Spectrometry Analysis. The AS and the 12 HPLC salivary fractions were analyzed by LC-ESI-MS and MALDI-TOF/TOF, respectively. The AS was analyzed by LC-ESI-MS with the HPLC analysis performed on a liquid chromatograph (Hewlett-Packard 1100 series) equipped with the same column referred previously. The solvents and the HPLC gradient used were the same as those reported above for the HPLC analysis. Double online detection was done in a photodiode spectrophotometer and by mass spectrometry. The mass detector was a Finnigan LCQ Deca (Finnigan Corporation, San Jose, CA) equipped with an API source, using an electrospray ionization (ESI) interface. Both the auxiliary and the sheath gases were a mixture of nitrogen and helium. The capillary voltage was 15 V and the capillary temperature 325 °C. Spectra were recorded in positive ion mode between m/z 250 and 2000 Da.

The 12 HPLC salivary fractions were analyzed by MALDI-TOF/TOF, using a 4800 MALDI-TOF/TOF analyzer (Applied Biosystems, Foster City, CA) in the linear mode to obtain the molecular weight of larger species and in the reflectron mode to obtain the peptide sequence of small species. In linear mode, all samples were mixed (1:1) with a matrix solution (3 mg/mL) of α-cyano-4-hydroxycinnamic acid matrix prepared in 50% ACN/0.1% TFA. Aliquots of samples (0.35 μL) were spotted onto the MALDI sample target plate, and spectra were obtained in the mass range between 1500 and 70000 Da with ca. 1000 laser shots.

Top Down Analysis. Characterization of smaller species present in each fraction was performed in the positive ion reflector mode using the above matrix composition. Each fraction was applied in triplicate and MS spectra were obtained in the mass range between 800 and 4500 Da with ca. 1200 laser shots. A fragmentation voltage of 2 kV was used for MS/MS analysis. Automated acquisition of MS and MS/MS data in the batch mode employed an interpretation method with the following settings: number of shots per spot =10; minimum S/N filter =50 to select peaks for MS/MS analyses, chromatogram peak width =3, and fraction resolution of precursor exclusion window =200 fwhm.

Peptide Mass Fingerprint (PMF). SDS−PAGE bands were digested with trypsin, and the generated tryptic peptides were analyzed. Peptide mass spectra were obtained on a MALDI-TOF/-TOF mass spectrometer (4800 Analyzer; Applied Biosystems, Foster CA) in conditions similar to those in the top-down analysis in the positive ion reflector mode automated acquisition of MS and MS/MS data. In this case, the interpretation method excludes the trypsin autolysis peaks.

Data Analysis. The spectra were processed and analyzed by the Global Protein Server Workstation (Applied Biosystems, Foster City, CA, USA), which uses internal Mascot software (v.2.1.0.4, Matrix Science Ltd., U.K.) for protein/peptide identification based on peptide mass fingerprints and MS/MS data.

The search was performed against the SwissProt protein database (march 2009, 428650 entries) for *Homo sapiens*. A MS tolerance of 30 ppm was found for precursor ions and 0.3 Da for fragment ions, as well as two missed cleavages. In the case of top-down, no enzyme was selected, and in the case of PMF, trypsin was selected. Protein identifications were considered as reliable when the MASCOT score was >70 (the MASCOT score was calculated as $-10 \times \log P$, where P is the probability that the observed match is a random event). This is the lowest score indicated by the program as significant ($P < 0.05$) and indicated by the probability of incorrect protein identification. In order to estimate the false discovery rate (FDR) and considering the repetitive PRP motif, a random decoy database was created for all SwissProt and internal database entries resulting in 10% of FDR (false positive peptides/(false positive peptides + total peptides)) × 100. Unique peptides retrieved from the FDR search were considered.

Protein−Tannin Interaction. The AS sample was analyzed by HPLC-DAD before and after the interaction with increasing concentrations of GSF. The control condition was a mixture of AS (150 μL) and acetate buffer 0.1 M, pH 5.0, and 12% ethanol (50 μL) (final volume 200 μL). For the experiments with GSF, the necessary volume (10 to 39 μL) of a GSF stock solution (2.66 mM) was added to AS (150 μL) to obtain the desired final concentration, plus the volume of acetate buffer to make the final volume 200 μL. GSF was tested in the following final concentrations: 0.133, 0.306, 0.399, 0.505, 0.599, 1.300 mM, and each concentration was an independent experiment. After shaking, the mixture reacted at room temperature (20 °C) for 5 min and then was centrifuged (8000 g, 5 min). The supernatant was injected into the HPLC-DAD.

These experiments were also made in large scale with two different concentrations of GSF 0.133 and 0.232 mM to do semipreparative HPLC-DAD. The 12 fractions were collected by recovering of the eluent deriving from the diode array detector. The 12 fractions were dried in a SpeedVac and analyzed by SDS−PAGE.

RESULTS AND DISCUSSION

In order to observe in vitro the effect of condensed tannins in quantitative and qualitative changes of the HPLC profile of human saliva, the first major task is the identification of the proteins corresponding to the HPLC profile. The HPLC method and identification described herein were based on the work developed by Messana et al.[30]

Several HPLC analytical problems may appear related to the quantity of mucins and other high molecular weight glycoproteins present in saliva. Indeed, these proteins can block and contribute to the degeneration of HPLC columns and poor reproducibility. In order to overcome these problems, saliva samples were mixed immediately after collection with aqueous TFA (final concentration 0.1%). This acidic treatment causes the precipitation of several high molecular weight SP (such as α-amylases, mucins, carbonic anhydrase, and lactoferrin) contributing to a decrease of the viscosity and also preserves sample

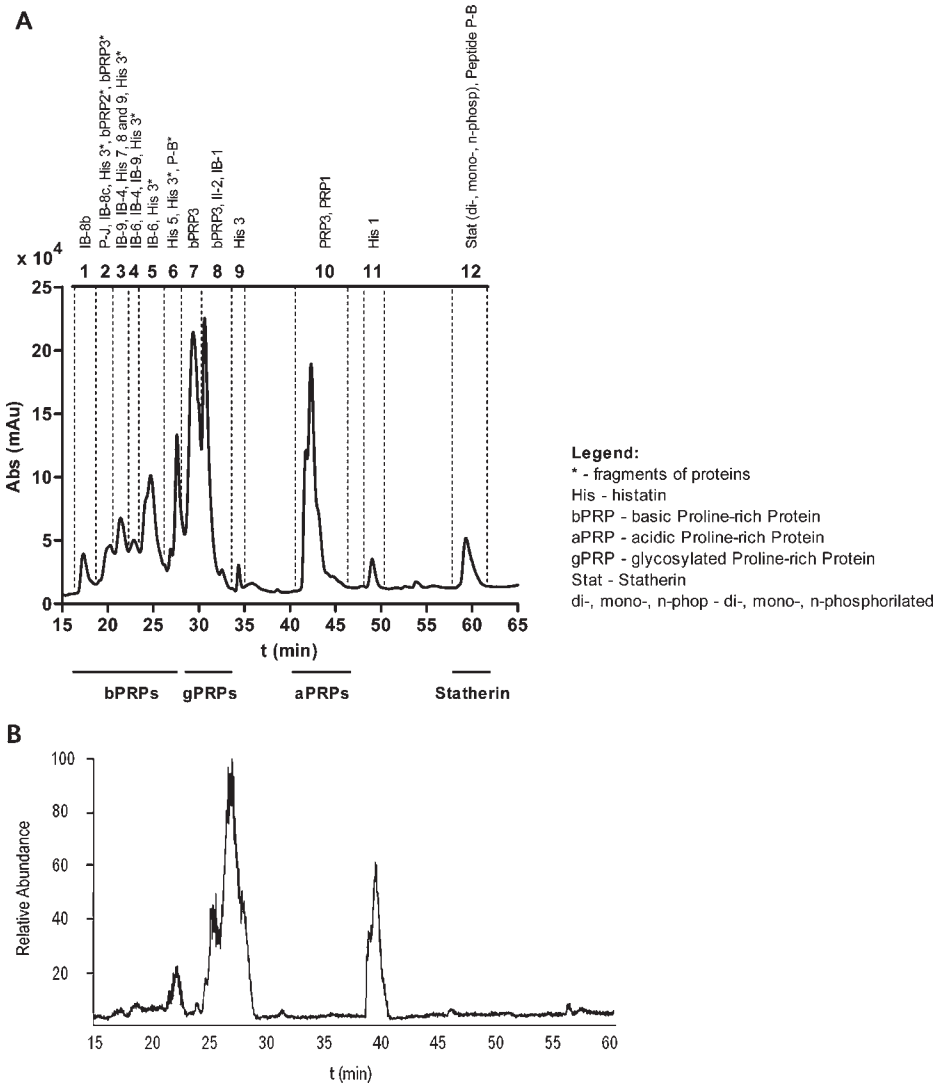

Figure 1. (A) Typical RP-HPLC profile detected at 214 nm of the acidic saliva (AS) solution of whole human saliva. The pointed lines and numbers show the ranges and names assigned to each HPLC fraction, with the outline of the main proteins identified for each HPLC fraction (Table 1). At the bottom, there is the distribution of the different families of salivary proteins along the chromatogram. *, fragments of proteins; His, histatin; Stat, sthaterin; (di-, mono-, *n*-)-phosp, (di-, mono-, non-)-phosphorylated; bPRP, basic proline-rich protein; gPRPs, glycosylated proline-rich proteins, aPRPs, acidic proline-rich proteins. (B) Total ion current (TIC) profile of AS solution collected by the ion-trap mass spectrometer (ESI-MS).

protein composition since TFA partially inhibits intrinsic protease activity.[30,58] Peptides and proteins such as histatins, basic and acidic proline-rich proteins (PRPs), statherin, cystatins, and defensins are soluble in acidic saliva (AS) solution and may be directly analyzed by RP-HPLC. The use of TFA provides a satisfactory compromise between high ion-pairing strength for chromatographic separation and protein ionization for ESI analysis. The HPLC chromatogram of this AS solution at 214 nm is presented in Figure 1A, and the profile was similar to the one previously described in the literature by Messana et al.[30]

On the basis of the identification of the different salivary proteins, the HPLC chromatogram of the AS solution was roughly divided into 12 peptide salivary fractions.

Identification of Salivary Proteins. In order to identify the main SP of each HPLC fraction, the AS solution was analyzed by several techniques such as RP-HPLC-ESI-MS, MALDI-TOF/TOF, and SDS−PAGE-MALDI-TOF/TOF. The total ion current (TIC) chromatogram profile obtained by HPLC-ESI-MS (Figure 1B) is similar to the chromatogram obtained at 214 nm (Figure 1A).

These identifications were first achieved by LC-MS analysis and deconvolution of the averaged ESI mass spectra. An example of the deconvolution process is presented in Figure 2 for the peak eluted at 40 min in Figure 1B.

In general, the deconvolution of the average TIC spectra of each area allowed the identification of 27 peptides shown in Table 1.

Table 1 reports all the proteins/peptides identified in the 12 HPLC fractions of the AS solution. It also indicates the experimental masses detected by RP-HPLC-ESI-MS, MS/MS, and MALDI-TOF (linear or after trypsin digestion). Using these different techniques, we were able to identify several peptides/small proteins. From the results presented in Table 1, it can be observed that the different techniques are complementary to identifying the major SP, probably because the peptides/proteins ionized differently according to the technique used.

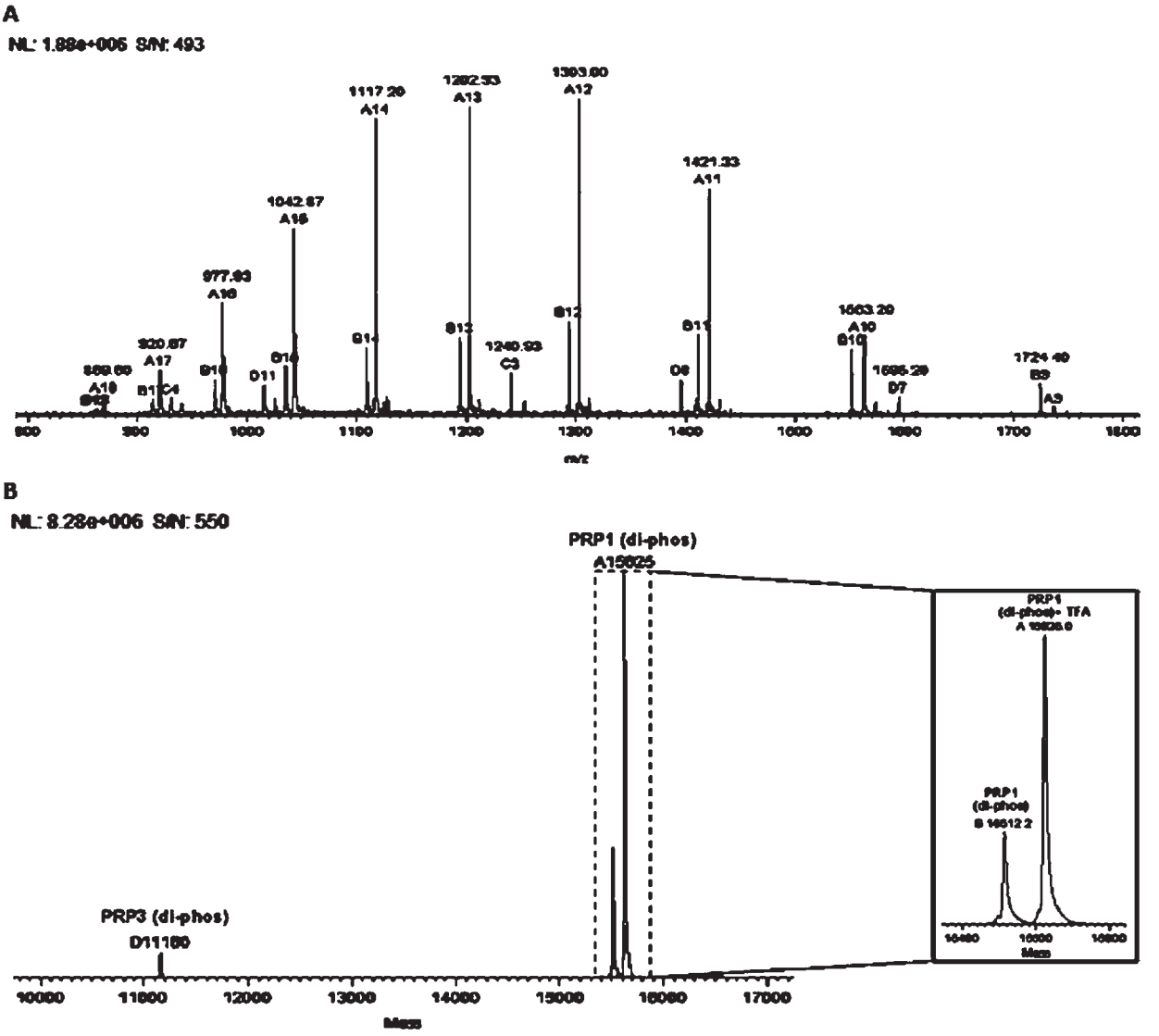

Figure 2. Deconvolution process for fraction 10. (A) ESI mass spectrum obtained by the average of 77 mass spectra collected in the 38.49−40.39 min range during the HPLC separation reported in the TIC profile (Figure 2). (B) The bottom panel reports the deconvolution of the upper ESI-MS (A).

The assignment of experimental masses to peptides/proteins was performed against the SwissProt protein database for *Homo sapiens* and also taking into account the previous identifications and experimental masses of these SP by ESI-MS and MALDI-TOF approaches by other groups, using similar experimental conditions.[30,59]

In order to confirm the assignment of some peptides performed by ESI-MS data and to increase the information of each HPLC fraction, the 12 chromatographic fractions displayed in Figure 1A were isolated individually by HPLC and analyzed by different approaches: (a) sequencing of peptides with MW below 5000 Da by direct MALDI-TOF MS/MS of each fraction, (b) SDS−PAGE of each fraction using different staining procedures, (c) identification of peptides/proteins with MW above 5000 Da by trypsin digestion of the bands after the SDS−PAGE analysis, and (d) linear MALDI-TOF analysis of each fraction. This strategy has been used by expert groups in this area.[30,59]

It is also important to refer that for glycosylated PRPs (gPRPs) the identification is even more difficult. The lack of the "total" molecular weight of these proteins including the sugar moiety makes the direct identification by ESI more complicated. Nevertheless, the digestion with trypsin and analysis by MALDI-TOF as well as Schiff's staining of the SDS−PAGE gels (see below) make it possible to identify a protein (bPRP3) belonging to the gPRPs class in HPLC fractions 7 and 8.

It is interesting to notice that the deconvolution of the ESI average spectra for HPLC fractions 7 and 8 gives a protein with a mass of 23467 Da, which could not be identified since there is no report in the databases or elsewhere in the literature. Messana et al. [30] have also identified a SP with this mass, but they were not able to identify this protein. However, they suggested that this unknown protein may be a putative basic PRP candidate since that protein was resistant to trypsin cleavage, which is a main characteristic of this kind of proteins.

The relative quantification of the proteins identified in each fraction based on the intensities of the peaks is inaccurate due to differences in ionization ability of the different proteins but could be a good approximative approach.

For the peptides identified by ESI-MS/MS, the extracted ion current (XIC) strategy was applied, which compares the ion

Table 1. Experimental Masses (Da) Detected in the Twelve Chromatographic Fractions by RP-HPLC-ESI-MS, and MALDI-TOF/TOF[a]

HPLC fraction	peptide	theoretical mass	Exp. ESI	Exp. MALDI*
1	**IB-8b**	4371	4369.9	4369.1
	bPRP2 $^{(379-407,379-408,379-412)}$			2817.3,2874.1,3271.1
	bPRP4$^{(261-305)}$			4336.7
	bPRP1$^{(236-279)}$		4392.9	4392.5
2	**His 3**$^{(33-43,24-36,20-36)}$			1434.5,1750.8,2161.1
	bPRP2$^{(379-412,368-407,368-408)}$			3271.3,3963.6,4020.6
	bPRP3$^{(268-288,219-261)}$			2027.8,4396.5
	P-J	5945	5942.9	5949.3
	IB-8c	5843	5841.5	5847.7
	bPRP1$^{(94-139,235-261)}$			2692.3,4376.5
	IB-8b	4371	4369.6	4369.2
	bPRP4$^{(263-307)}$			4398.5
	His 8	1562	1561.7	1562.7
	His 10	1719		1718.8
3	**IB-9**	6023	6023.3	6029.9
	IB-4	5590	5589.6	5593.5
	His 7	1718		1718.8
	His 3$^{(42-51,30-43,20-36,20-37,20-39)}$			1264.5,1846.9,2161.1,2298.0,2522.1
	His 8	1562	1561.7	1562.7
	His 9	1875		1874.9
4	**His 3**$^{(24-33,20-33,30-43)}$			1356.7,1766.8,1846.9
	IB-5	6950	6949.1	6957.4
	IB-9	6023	6022.7	6028.2
	IB-4	5590		5593.1
5	**His 3**$^{(20-33,37-51,29-43,26-43,24-41,20-41)}$			1766.9,1918.8,2009.9,2341.1,2405.2,2815.3
	IB-6	11517	11515.6	
	peptide P-B $^{(57-67)}$			1161.5
6	**His 5**	3036	3036.6	3035.3
	His 3$^{(24-43,\ 34-51,24-44)}$		2625.0	2625.1, 2312.8, 2781.2
	peptide P-B$^{(55-67)}$			1315.6
	His 6	3192	3192.0	3191.4
7	**bPRP3**			
	unknown		23467.0	
	His 3$^{(55-69,33-51)}$			1920.8, 2459.9
	peptide P-B$^{(55-67,55-69)}$			1315.7, 1469.7
8	**bPRP3**			
	unknown		23467.0	
	II-2 (phos.)	7608	7607.1	7613.7
	IB-1	9590	9590.7	
	His 3$^{(33-51)}$			2459.9
	peptide P-B$^{(68-79)}$			1200.6
9	**His 3**	4061	4061.0	4061.0
10	**PRP1 (diphos)**	15514	15512.0	
	PRP3 (diphos)	11161	11160.0	
	Cys S		14347.0	
	Cys SN			

Table 1. Continued

HPLC fraction	peptide	theoretical mass	Exp. ESI	Exp. MALDI*
11	**His 1**	4926	4927.3	4932.1
	His 1$^{(37-57,34-57,33-57,32-57)}$			2617.9,3012.1,3159.2,3287.3
	His 2$^{(31-57)}$	3443	3441.4	3443.6
12	**statherin (diphos)**	5378	5378.7	5381.5
	statherin (monophos)	5299		5299.9
	statherin (n-phos)	5219		5216.9
	peptide P-B	5789	5791.2	5796.3

[a] The experimental masses obtained by ESI-MS were compared to the theoretical masses of human salivary proteins reported in international data banks, and the experimental masses obtained by MALDI-TOF/TOF were identified as referred to in the Materials and Methods section. The superscripted numbers are the residue numbers for the peptides identified for each protein; the proteins in bold are the main proteins for each fraction. Exp. ESI, experimental masses obtained in ESI analysis; * masses obtained by MS/MS and linear MALDI. SwissProt code: IB-8b, PRP1 and PRP3 (P02810); IB-8c and bPRP2 (P02812); bPRP1, P-J, IB-4, IB-6 and II-2 (P04280); bPRP3 (Q04118); bPRP4 and IB-5 (P10163); IB-9 (P02811); IB-1 (P04281); peptide P-B (P02814); His 3, 5, 6, 7, 8, 9, and 10 (P15516); His 1 (P15515); Cys S (P010136); Cys SN (P01037); statherin (P02808).

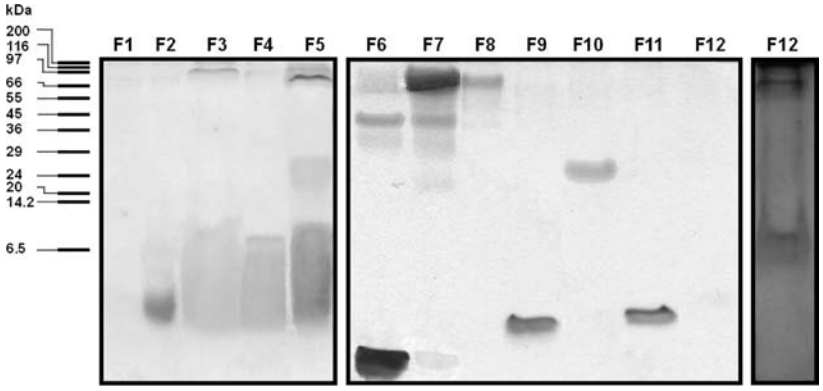

Figure 3. SDS−PAGE of the 12 HPLC fractions isolated from the HPLC after the injection of the AS solution. The molecular weight markers were substituted by lines, and the molecular mass is expressed in kDa as marked on the left side. The gels were stained by with Imperial Protein Stain, a Coomassie R-250 dye-based reagent.

currents originated by the peptides. The deconvolution process considers the major ions of ESI spectra giving the relative quantity of each protein identified. On the basis of that approach, the major proteins of each HPLC fraction were identified and are indicated with bold text in Table 1.

From the results presented, it is possible to observe that several proteins were found, namely, IB-1, IB-8b, IB-4, IB-9, IB-5, IB-6, bPRP3, II-2, PRP1, PRP3, histatin 5, 7, 8, and several forms of statherin; small fragments were assigned as histatin 3, bPRP1, bPRP2, and bPRP3 fragments. These observations had been previously reported by Vitorino et al.[59]

In summary, the chromatogram is roughly divided into four regions corresponding to the different families of SP. The first zone (1 to 6) comprises proteins that belong to the classes of bPRPs and His. The second region (7 and 8) comprises mainly a gPRP, the bPRP3. The third region (10) has as main proteins the aPRPs. The last region (12) has phosphorylated and nonphosphorylated forms of statherin.

SDS−PAGE. In order to obtain additional information and to do trypsin digestion of the SP of each HPLC fraction, SDS−PAGE of each fraction was carried out, and the results are presented in Figure 3.

Although SP can be separated and analyzed by SDS−PAGE, there are some particular aspects that have to be considered, especially regarding PRPs. PRPs stain poorly with conventional Coomassie Blue and even with silver staining procedures. However, when Coomassie Blue R-250 is used and organic solvents are omitted from the destain solution, PRPs stain pink−violet; regarding silver staining, some improved staining can be achieved by a modified silver staining procedure. Nevertheless, for PRPs the sensitivity is lower than that for other proteins.[60] From the results presented in Figure 3, it is possible to observe that for fractions 1 (F1) and 12 (F12) there were no bands detected, even after testing different sample concentrations and different staining methods with these fractions.

It is also necessary to be cautious with the apparent molecular weight of this kind of proteins on SDS−PAGE gels; several reports have described that PRPs do not migrate in SDS−PAGE at the expected molecular weight.[61,62] The high proline content presumably increases the rigidity of the protein, which makes the protein migrate slower in SDS−PAGE than globular proteins with the same molecular weight (MW).

Unusual mobility on SDS−PAGE is one of the characteristics of intrinsically unstructured proteins. Because of their unusual amino acid composition, PRPs bind less SDS than usual, and their apparent MW on SDS−PAGE gel is often 1.2−1.8 times higher (MW factor) than the real one.[61]

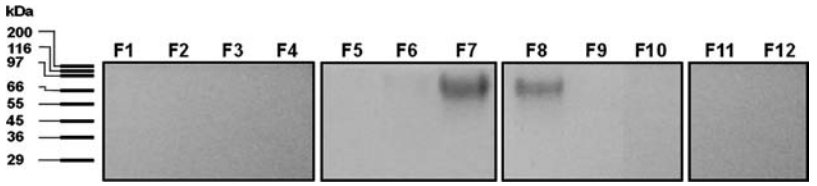

Figure 4. SDS−PAGE of each HPLC fraction isolated from the HPLC after the injection of the AS solution. The molecular weight markers were substituted by lines and the molecular mass is expressed in kDa. The gels were stained by the periodic acid Schiff procedure in order to visualize glycoproteins.

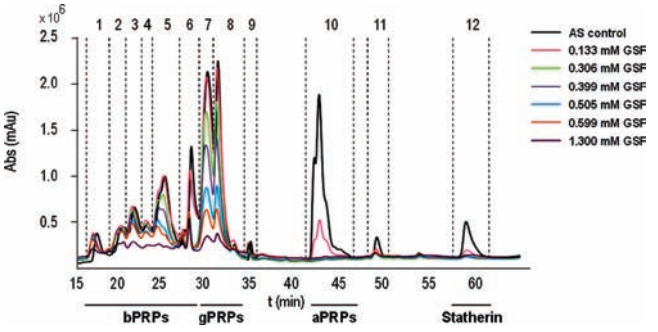

Figure 5. RP-HPLC profile detected at 214 nm of the AS solution before (AS control) and after the interaction with increasing concentrations of grape seed fraction (GSF).

For fraction 2 (F2), a major band smaller than 6.5 kDa is visible, which could correspond to several peptides previously identified by ESI-MS and MALDI-TOF-MS for this fraction, peptides deriving from histatin 3, bPRP2, and bPRP3 with a molecular weight between 1.4 and 4.4 kDa. Besides these, peptides P-J and IB-8c with a molecular weight of 5.9 and 5.8 kDa, respectively, were also identified for this fraction. These peptides could correspond to a small smear just below the 6.5 kDa marker.

For fraction 3, it is possible to observe a significant smear at the bottom of the gel and a high molecular weight band. The smear at the bottom of the gel spreads below the 6.5 kDa. The upper part of the smear could correspond to IB-9 and IB-4 proteins. These proteins have molecular weights of 6.0 and 5.5 kDa, respectively, and they could migrate in the area of the 6.5−7.0 kDa (in this case, the MW factor is 1.2). However, the other identified peptides, histatins 7, 8, 9, and fragments of histatin 3, have lower molecular weight around 2.1 kDa, and therefore, they will migrate at the bottom of the gel. Regarding the band at the top of the gel, its identification was not possible.

For fraction 4 (F4), the results presented are quite similar to F3. There is a significant smear at the bottom of the gel. At the top of the smear, there is probably IB-5, IB-9, and IB-4 with molecular weights of 6.9, 6.0, and 5.6 kDa, respectively. These proteins could migrate in the area of 6.7 to 8.0 kDa (MW factor of 1.2).

For fraction 5 (F5), it is possible to observe a pink−violet band in the region of 20 kDa. This band could be IB-6 protein, which was identified in this fraction by deconvolution of the average ESI. Indeed, the real MW is 11.5 kDa, but as referred to above, it could migrate with an apparent MW of 11.5 × 1.8 (MW factor) = 20.7 kDa. This identification is further supported by the pink−violet color (characteristic of PRPs) displayed by this band. This fraction presents one smear at the bottom of the gel. At the bottom of this smear could be the several fragments of histatin 3 previously identified (MW around 2.0 kDa), but there were no identified peptides/proteins around 6.5 kDa. Therefore, the upper part of the smear, as well as the band at 66 kDa, was not identified.

Fraction 6 (F6) presents two important bands. The analysis of the intense low molecular weight band revealed the presence of histatin 5 (3.0 kDa) and several fragments of histatin 3 at 2.5 kDa. The other band at the region of 45 kDa is probably one protein that appears in the ESI analysis with 23.4 kDa (will migrate with an apparent MW of 42 kDa). This protein seems also to be eluted in fraction 7 collected from the HPLC. Messana et al.[30] have also verified the existence of that protein, and they suggested that it could correspond to basic PRPs.

For fractions 7 (F7) and 8 (F8), there is a main band at the top of the gel with an apparent MW of around 66 kDa. The analysis of this band by MALDI-TOF after trypsin digestion allowed the identification of bPRP3 protein, a glycosylated bPRP. The presence of sugars in protein structure was confirmed by periodic acid schiff (PAS) staining, which is a basic procedure for the analysis of glycoproteins (Figure 4). Basically, this procedure stains the sugar moieties of glycoproteins yielding magenta bands with a colorless background.

Only these two fractions gave positive results to the PAS staining, which indicates that glycosylated proteins are eluted only in those two fractions (Figure 4).

Although two other proteins, namely, II-2 and IB-1, have been identified in F8 by ESI deconvolution, they do not appear in the SDS−PAGE gel probably because their quantity is below the detection limit of the Coomassie stain.

For fraction 9 (F9), only histatin 3 (4.0 kDa) was identified in the SDS−PAGE gel.

Fraction 10 (F10) presents one band with an apparent MW of 24−29 kDa. The previous analysis of this fraction by ESI-MS indicated the presence of two aPRPs, PRP1 and PRP3 (15.5 and 11.2 kDa, respectively). Considering the factor 1.8 into the apparent MW, we determined that these proteins would appear in the SDS−PAGE gel in the zone of 20−27 kDa, which is in agreement with the results obtained.

The band observed at the bottom of the SDS−PAGE gel of fraction 11 (F11) could be attributed to the identified histatin 1 (4.9 kDa).

For fraction 12 (F12), different isoforms of statherin were identified, namely, di (5.4 kDa)-, mono (5.3 kDa)-, and nonphosphorylated (5.2 kDa) forms. The peptide P-B (5.8 kDa) was also identified, but surprisingly no bands were detected in the SDS−PAGE gel stained with a Coomassie based dye. However, the silver staining revealed the presence of a smear in the 6.5 kDa region; probably this smear corresponds to the several isoforms of statherin and to the peptide P-B since that these proteins have approximately the same molecular weight.

Interaction between Salivary Proteins and Condensed Tannins. The experiments in this study were all performed in buffer with 12% ethanol to mimic a model wine and at pH of 5.0

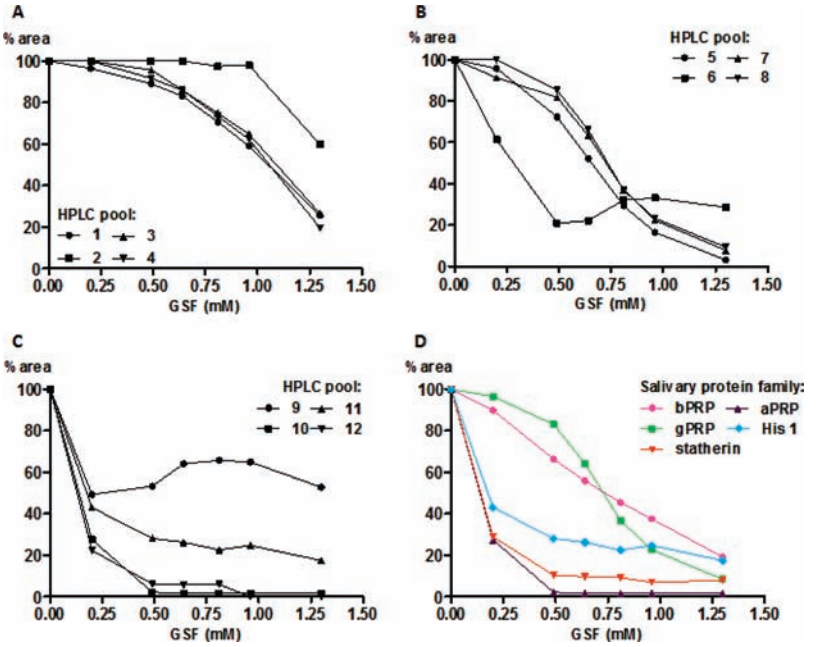

Figure 6. Percentages of area decrease of each HPLC fraction after the interaction of AS solution with increasing concentrations of GSF (A, B, and C). Percentages of area decrease for each family of salivary proteins (D).

as has already been referred to as a pH at which salivary proteins strongly interact with condensed tannins[63] and correspond to a intermediary pH between wine pH (3.4) and saliva pH (7.0). Indeed, salivary pH drops with the ingestion of acidic drinks, and the degree of acidity in saliva depends on the sampled volume, buffering capacity, and mode of drinking.[64,65] In order to compare the reactivity of the identified proteins with condensed tannins, different concentrations of grape seed fraction (GSF) were mixed with acidic saliva (AS) solution, and after centrifugation, the supernatant was analyzed by HPLC. The profiles of AS solution before and after the interaction with GSF are shown in Figure 5.

The results presented in Figure 5 show that the HPLC profile of the AS solution is interestingly affected by the interaction with GSF. Effectively, GSF interacts in a different way with the different groups of proteins. While the areas of HPLC fractions 10 and 12 declines significantly with the lowest GSF concentration, the other fractions' areas remain relatively constant. It is important to mention that the mixture of AS solution with GSF always resulted in the formation of insoluble precipitates, which increased along with GSF concentration.

The decrease of percentage area for each HPLC fraction after the AS solution interaction with increasing concentrations of GSF is summarized in Figure 6.

From the results presented in Figure 6, it is possible to observe that fractions 10, 11, and 12 interact more importantly with GSF (Figure 6C). For the lowest GSF concentration (0.133 mM) assayed, the area of these fractions was much reduced to 20% for fractions 10 and 12 and to 40% for fraction 11. With the increase in GSF concentration (0.505 mM), fractions 5 and 6 were also greatly reduced to 30% (Figure 6B). However, regarding the fractions 1 to 4 and 9, only higher GSF concentrations significantly reduced their areas (Figure 6A and C). The results were gouped by SP families (Figure 6D), and it is possible to observe that the major SP families that interact with GSF were aPRPs and statherin. For the SP families gPRPs and bPRPs, their interaction depends on the concentration of GSF. For the lowest concentration of GSF, the family that interacts less with GSF is gPRPs. However, increasing the concentration of GSF leads to a more significant interaction with gPRPs. Among the three main families of SP, for the highest GSF concentration, bPRPs are the ones that interact less with GSF.

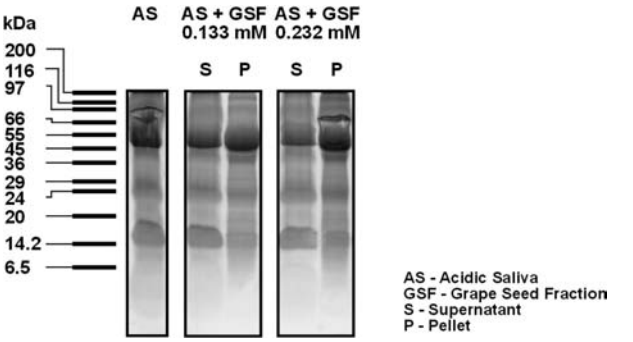

Figure 7. SDS−PAGE of the AS solution before and after the interaction with two concentrations of grape seed fraction (GSF) (0.133 mM and 0.232 mM). The molecular weight markers were substituted by lines, and the molecular mass is expressed in kDa.

In order to obtain additional information about which SP of each HPLC fraction is interacting with tannins, SDS−PAGE of AS before and after the interaction with GSF was carried out. The resulting precipitate was also analyzed by SDS−PAGE, which is widely used for the analysis of human fluids containing proteins as well as for the study of tannin−protein interactions.[41] In fact, gel electrophoresis is a useful tool for assessing tannin binding proteins in human saliva because the proteins dissociate from the insoluble tannin−protein complexes in the presence of SDS and can be visualized and identified in the gel.

Figure 7 presents the results of the SDS−PAGE of AS before and after the interaction with 0.133 and 0.232 mM of GSF.

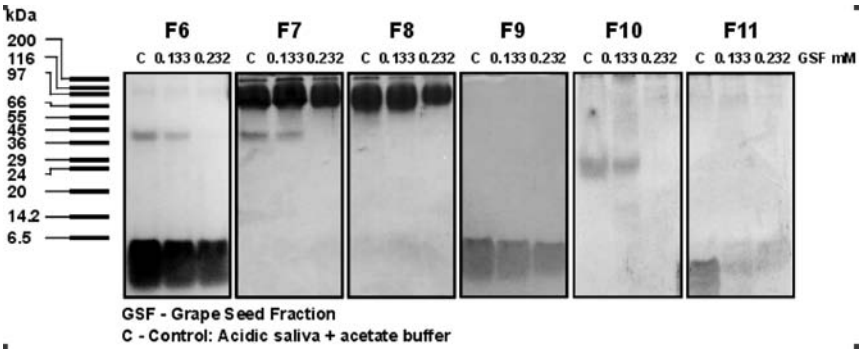

Figure 8. SDS−PAGE of HPLC fractions 6 to 11 isolated from the HPLC after the injection of the AS solution before (C) and after the interaction with two concentrations of grape seed fraction (0.133 and 0.232 mM). The molecular weight markers were substituted by lines, and the molecular mass is expressed in kDa.

The results presented in Figure 7 clearly show that the proteins existing in the control AS precipitate by GSF. It is also perceptible that increasing GSF concentration also increases the amount of SP that appears in the respective precipitate.

The HPLC fractions that previously showed a significant interaction with GSF (Figures 5 and 6), namely, fractions 5 to 12, were also analyzed by SDS−PAGE after the interaction with GSF. The same saliva sample was incubated with two different concentrations of GSF (0.133 and 0.232 mM) and centrifuged, and the supernatant was analyzed by HPLC. After HPLC collection, the fractions were dried in a SpeedVac, dissolved in the same control volume, and analyzed by SDS−PAGE. The results are presented in Figure 8. The gels for F5 and F12 are absent because it was not possible to see any band in the control assay.

From the results presented, it is possible to see that for F6 the band that appears at 45 kDa, probably corresponding to a bPRP as previously referred, and disappears with the increase in GSF concentration. Similar behavior was observed for the same protein that also appears in F7.

The intensity of the band of bPRP3 (near the 66 kDa) in F7 decreases slightly with increasing GSF concentration. However, the effect of GSF in this protein/band is more visible in F8. In general, these results are in agreement with the ones obtained in the HPLC analysis (Figure 5); for F7 and F8, only with GSF concentration above 0.5 mM is the profile greatly reduced. For the lowest GSF concentrations, the HPLC profile of these fractions is not very affected.

The band corresponding to aPRPs in F10 disappear after 0.232 mM GSF concentration. This result is in agreement with the results obtained from the HPLC analysis (Figure 5). Furthermore, the band's intensity corresponding to histatin 3 and 1 in F9 and F11, respectively, is reduced importantly with the increase in GSF concentration. Overall, the main family of SP that disappears considerably after GSF incubation is the aPRPs (PRP1 and PRP3) present in HPLC fraction 10.

These results seem to indicate that statherin and especially acidic PRPs (aPRPs) have a high relative affinity toward condensed tannin complexation compared to that of the other SP, in a competitive assay at pH 5.0. The acidic proteins PRP1 and PRP3 have 150 and 106 aminoacid residues, respectively. The first 106 residue sequence from PRP1 corresponds to PRP3. The acidic character of these proteins is confined roughly to the first 30 amino acids at the N-terminal due to the presence of many aspartic and glutamic acid residues. The remaining part is basic and, similarly to basic PRPs, shows repeated sequences of proline and glutamine.[22] In general, PRPs have open randomly coiled structures. These open structures allow the exposure of peptide carbonyl groups to hydrogen bonding as well as the exposure of proline residues to act as binding sites for tannin by hydrophobic interaction of the aromatic portion of tannin with the pyrrolidine structure of proline. Nevertheless, in the case of aPRPs, previous studies on calcium binding to aPRPs pointed to an interaction between the N- and C-terminal regions of the proteins (possibly by electrostatic forces) indicating that the structures are not so open when compared to those of other PRPs.[66,67] However, at pH 5.0 as studied herein, which is close to the isoelectric point (pI) of PRP1 and PRP3 (4.63 and 4.14, respectively), several acidic amino acids in the N- terminal part are not expected to be charged, reducing the interaction with C-terminal and opening the structure of aPRPs.[42] Consequently, proline residues are readily accessible to promote hydrophobic interaction with tannins. However, the presence of an important number of carboxyl groups in aspartic and glutamic amino acid residues in the N-terminal part may contribute importantly to strengthen the interaction with tannins through hydrogen bonds.

Concerning the basic PRPs (bPRPs), these results generally show a significant decrease of their area but only for the highest GSF concentrations (0.599 and 1.3 mM) and when almost all the other proteins have been depleted. These results seem to indicate that the bPRPs have a low relative affinity toward condensed tannin complexation compared to that of the other SP, in a competitive assay at pH 5.0. Not totally in agreement with this, Lu and Bennick[35] have measured the amount of condensed tannins (crude quebracho tannin) and tannic acid precipitated by pure bPRPs (IB-1, IB-4, IB-8b, and IB-6), aPRPs (PIF-s), and gPRPs (gPRPs) at pH 7.4. These authors have observed that each bPRP precipitated a higher quantity of tannins compared to that of the other PRPs. Also, they showed that there were only small differences in the tannin-precipitating ability of various bPRPs of different sizes or sequences, indicating that, although there is considerable phenotypic variation of PRPs, it is not likely to cause marked individual variation in tannin-binding ability. However, it is difficult to compare the results obtained by these authors with the ones obtained herein because first of all, the tannins used are completely different from ours, being a complex mixture and not properly characterized. Also, these authors measured the quantity of tannin precipitated, which depends not only on the PRP affinity but also on the stoichiometry of the complexes. However, the pH used by these authors (pH 7.4) and herein (pH 5.0) are substantially different, which will probably differently affect the interaction for the reasons mentioned above.

The interaction of GSF with phosphorylated forms of statherin (HPLC fraction 12) is also important. The results yielded by the HPLC analysis show a significant interaction with statherin being one of the main SPs that interacts with condensed tannins in the conditions described here. According to our knowledge, only Nayak and Carpenter[68] showed that statherin is precipitated by polyphenols, namely, tea polyphenols. Statherin is abundant in tyrosine residues and is phosphorylated at Ser2 and Ser3. Several variants have been identified and are derived both by alternative splicing and posttranslational modifications. The presence of protic groups (phosphates) with acidic character may favor the hydrogen bond interactions which probably explain statherin's high affinity to tannins.

Regarding the glycoproteins, bPRP3 did not show a high interaction at lowest GSF concentration. Nevertheless, increasing GSF concentration leads to a significant decrease of that protein. For GSF concentration of 1.3 mM, the area of HPLC fractions 7 and 8 has been decreased about 60%, while other HPLC fractions (bPRPs 1 to 6) only decreased about 40%. As previously mentioned, bPRP3 is a glycosylated protein that contains about 50% carbohydrate, which is composed of highly fucosylated N-linked saccharides.[69] Glycosylated PRPs (gPRPs), which have been implicated in the oral epithelium lubrication,[70] were shown here to resist precipitation compared to that of aPRPs, histatins, and statherin. Sarni-Machado et al.[46] have also observed that low concentrations of condensed tannins (mainly (+)-catechin, (−)-epicatechin, and epicatechin gallate) first precipitate lower molecular weight SP and only for higher concentrations precipitate glycosylated PRPs. More recently, the same authors[14] have observed that the glycosylation of human PRPs favors the formation of soluble complexes and reduces tannin precipitation with regard to tannin amounts. This is in agreement with Pascal et al.[15] who have stated that protein glycosylation (gPRP, similar to II-1 herein) prevented PRP-condensed tannin precipitation when compared to that of a nonglycosylated PRP (IB-5). These authors have concluded that gPRPs are effective in binding tannins but that these interactions do not necessarily result in precipitation. In conclusion, although gPRPs are not readily precipitated by condensed tannins compared with other SP, they can form complexes with tannins modifying the rheological properties of saliva such as viscosity and consequently astringency.[15]

The GSF also interacts importantly with histatin 1 (HPLC fraction 11), as shown both by HPLC analysis and by SDS−PAGE. The results also showed an interaction of tannins with histatins 3 and 5 (HPLC fractions 6 and 9), although not as significant as the one with histatin 1. Contrary to these results, Naurato and co-workers[71] have found that His 1 bound only about half the amount of condensed tannin (crude quebracho tannin and epigallocatechin gallate) than histatins 3 and 5, and there was no difference between these two latter in their ability to precipitate condensed tannins. Moreover, they found that histatins 3 and 5 share the same condensed tannin-binding region, with more tannin binding to the C-terminal region. However, all the experimental conditions and methodology used were quite different, namely, pH (7.4), buffer (isotonic barbital buffer), and temperature of interaction (37 °C).

Regarding the histatin (His) group, their interaction with tannins is well known.[42,71] They comprise a group of structurally related, small histidine-rich proteins found only in the saliva of humans and some monkeys. Twelve His, named His 1 to 12, have been isolated from human saliva and their primary structures determined.[21] The most prominent members are His 1, 3, and 5, and they account for 85−90% of this family. Histidine is the prominent amino acid, accounting for about 25% of all residues and, together with basic amino acids, makes up 30% to 75% of total amino acids. In contrast to PRPs, they contain no proline residues except for a single residue in His 1. It is interesting that, among the identified histatins, it was His 1 that was the most effective in binding polyphenols.

In conclusion, regarding the interaction between tannins and different families of SP, the published data at present is somehow controversial. Some authors have stated that all salivary PRP families have similar affinities toward different condensed tannins at a molecular level by means of a competition assay, while others stated that basic PRPs are the main family of SP that interact with condensed tannins. However, the experimental conditions described in the literature are often different, and the results should be analyzed carefully.

The results present herein provide important insights concerning the influence of the different families of SP in the development of astringency. In fact, when all the main families of SP are present in a competitive assay, like in the oral cavity, they demonstrate that tannins interact first with aPRPs and statherin and also interact significantly with histatins and gPRPs. For future experiments, it would be interesting to evaluate the interaction between condensed tannins and saliva from different donors and also to evaluate the interaction with hydrolyzable tannins.

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ABBREVIATIONS USED

PRPs, proline-rich proteins; bPRPs, basic proline-rich proteins; aPRPs, acidic proline-rich proteins; gPRPs, glycosylated proline-rich proteins; SP, salivary proteins; GSF, grape seed fraction; AS, acidic saliva; DLS, dynamic light scattering; PMF, peptide mass fingerprint; TFA, trifluoracetic acid; MW, molecular weight; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

REFERENCES

(1) Faria, A.; Calhau, C.; De Freitas, V.; Mateus, N. Procyanidins as antioxidants and tumor cell growth modulators. *J. Agric. Food Chem.* **2006**, *54*, 2392–2397.

(2) Koleckar, V.; Kubikova, K.; Rehakova, Z.; Kuca, K.; Jun, D.; Jahodar, L.; Opletal, L. Condensed and hydrolysable tannins as antioxidants influencing the health. *Mini-Rev. Med. Chem.* **2008**, *8*, 436–447.

(3) De La Iglesia, R.; Milagro, F. I.; Campión, J.; Boqué, N.; Martínez, J. A. Healthy properties of proanthocyanidins. *Biofactors* **2010**, *36*, 159–168.

(4) Jambunathan, R.; Mertz, E. T. Relation between tannin levels, rat growth, and distribution of proteins in sorghum. *J. Agric. Food Chem.* **1973**, *21*, 692–696.

(5) Goncalves, R.; Soares, S.; Mateus, N.; De Freitas, V. Inhibition of trypsin by condensed tannins and wine. *J. Agric. Food Chem.* **2007**, *55*, 7596–7601.

(6) Mcmanus, J. P.; Davis, K. G.; Lilley, T. H.; Haslam, E. The association of proteins with polyphenols. *J. C. S. Chem. Commun.* **1981**, 309–311.

(7) Bate-Smith, E. C. Astringency in foods. *Food* **1954**, *23*, 124.

(8) Goldstein, J. L.; Swain, T. Changes in tannins in ripening fruits. *Phytochemistry* **1963**, *2*, 371–383.

(9) Dinnella, C.; Recchia, A.; Fia, G.; Bertuccioli, M.; Monteleone, E. Saliva characteristics and individual sensitivity to phenolic astringent stimuli. *Chem. Senses* **2009**, *34*, 295–304.

(10) Schwarz, B., Hofmann, T. Is there a direct relationship between oral astringency and human salivary protein binding? *Eur. Food Res. Technol.* **2008**.

(11) Obreque-Slier, E.; López-Solís, R.; Peña-Neira, Á.; Zamora-Marín, F. Tannin−protein interaction is more closely associated with astringency than tannin−protein precipitation: Experience with two oenological tannins and a gelatin. *Int. J. Food Sci. Technol.* **2010**, *45*, 2629–2636.

(12) Dinnella, C.; Recchia, A.; Vincenzi, S.; Tuorila, H.; Monteleone, E. Temporary modification of salivary protein profile and individual responses to repeated phenolic astringent stimuli. *Chem. Senses* **2010**, *35*, 75–85.

(13) Bajec, M. R.; Pickering, G. J. Astringency: Mechanisms and perception. *Crit. Rev. Food Sci. Nutr.* **2008**, *48*, 858 –875.

(14) Sarni-Manchado, P.; Canals-Bosch, J. M.; Mazerolles, G.; Cheynier, V. Influence of the glycosylation of human salivary proline-rich proteins on their interactions with condensed tannins. *J. Agric. Food Chem.* **2008**, *56*, 9563–9569.

(15) Pascal, C.; Poncet-Legrand, C.; Cabane, B.; Vernhet, A. Aggregation of a proline-rich protein induced by epigallocatechin gallate and condensed tannins: Effect of protein glycosylation. *J. Agric. Food Chem.* **2008**, *56*, 6724–6732.

(16) Fischer, U.; Boulton, R. B.; Noble, A. C. Physiological factors contributing to the variability of sensory assessments: Relationship between salivary flow rate and temporal perception of gustatory stimuli. *Food Qual. Pref.* **1994**, *5*, 55–64.

(17) Payne, C.; Bowyer, P. K.; Herderich, M.; Bastian, S. E. P. Interaction of astringent grape seed procyanidins with oral epithelial cells. *Food Chem.* **2009**, *115*, 551–557.

(18) Mandel, I. D. Relation of saliva and plaque to caries. *J. Dent. Res.* **1974**, *53*, 246–266.

(19) Helmerhorst, E. J.; Oppenheim, F. G. Saliva: A dynamic proteome. *J. Dent. Res.* **2007**, *86*, 680–693.

(20) Schlesinger, D. H.; Hay, D. I.; Levine, M. J. Complete primary structure of statherin, a potent inhibitor of calcium phosphate precipitation, from the saliva of the monkey, macaca arctoides. *Int. J. Pept. Protein. Res.* **1989**, *34*, 374–380.

(21) Oppenheim, F. G.; Xu, T.; Mcmillian, F. M.; Levitz, S. M.; Diamond, R. D.; Offner, G. D.; Troxler, R. F. Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on candida albicans. *J. Biol. Chem.* **1988**, *263*, 7472–7477.

(22) Hay, D. I.; Bennick, A.; Schlesinger, D. H.; Minaguchi, K.; Madapallimattam, G.; Schluckebier, S. K. The primary structures of six human salivary acidic proline-rich proteins (prp-1, prp-2, prp-3, prp-4, pif-s and pif-f). *Biochem. J.* **1988**, *255*, 15–21.

(23) Shomers, J. P.; Tabak, L. A.; Levine, M. J.; Mandel, I. D.; Ellison, S. A. Characterization of cysteine-containing phosphoproteins from human submandibular-sublingual saliva. *J. Dent. Res.* **1982**, *61*, 764–767.

(24) Kauffman, D.; Wong, R.; Bennick, A.; Keller, P. Basic proline-rich proteins from human parotid saliva: Complete covalent structure of protein ib-9 and partial structure of protein ib-6, members of a polymorphic pair. *Biochemistry* **1982**, *21*, 6558–6562.

(25) Isemura, S.; Saitoh, E.; Sanada, K. Fractionation and characterization of basic proline-rich peptides of human parotid saliva and the amino acid sequence of proline-rich peptide p-e. *J. Biochem.* **1982**, *91*, 2067–2075.

(26) Isemura, S.; Saitoh, E.; Sanada, K. The amino acid sequence of a salivary proline-rich peptide, p-c, and its relation to a salivary proline-rich phosphoprotein, protein c. *J. Biochem.* **1980**, *87*, 1071–1077.

(27) Bennick, A. Chemical and physical characteristics of a phosphoprotein from human parotid saliva. *Biochem. J.* **1975**, *145*, 557–567.

(28) Azen, E. A.; Oppenheim, F. G. Genetic polymorphism of proline-rich human salivary proteins. *Science* **1973**, *180*, 1067–1069.

(29) Inzitari, R.; Cabras, T.; Onnis, G.; Olmi, C.; Mastinu, A.; Sanna, M. T.; Pellegrini, M. G.; Castagnola, M.; Messana, I. Different isoforms and post-translational modifications of human salivary acidic proline-rich proteins. *Proteomics* **2005**, *5*, 805–815.

(30) 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.

(31) Bennick, A.; Connell, G. E. Purification and partial characterization of four proteins from human parotid saliva. *Biochem. J.* **1971**, *123*, 455–464.

(32) Oppenheim, F. G.; Hay, D. I.; Franzblau, C. Proline-rich proteins from human parotid saliva. I. Isolation and partial characterization. *Biochemistry* **1971**, *10*, 4233–4238.

(33) Amado, F.; Lobo, M. J. C.; Domingues, P.; Duarte, J. A.; Vitorino, R. Salivary peptidomics. *Expert Rev. Proteomics* **2010**, *7*, 709–721.

(34) Henskens, Y. M. C.; Veerman, E. C. I.; Mantel, M. S.; Van Der Velden, U.; Nieuw Amerongen, A. V. Cystatins s and c in human whole saliva and in glandular salivas in periodontal health and disease. *J. Dent. Res.* **1994**, *73*, 1606–1614.

(35) Lu, Y.; Bennick, A. Interaction of tannin with human salivary proline-rich proteins. *Arch. Oral Biol.* **1998**, *43*, 717–728.

(36) Pascal, C.; Paté, F.; Cheynier, V.; Delsuc, M.-A. Study of the interactions between a proline-rich protein and a flavan-3-ol by nmr: Residual structures in the natively unfolded protein provides anchorage points for the ligands. *Biopolymers* **2009**, *91*, 745–756.

(37) Soares, S.; Mateus, N.; De Freitas, V. Interaction of different polyphenols with bovine serum albumin (bsa) and human salivary α-amylase (hsa) by fluorescence quenching. *J. Agric. Food Chem.* **2007**, *55*, 6726–6735.

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

(39) Monteleone, E.; Condelli, N.; Dinnella, C.; Bertuccioli, M. Prediction of perceived astringency induced by phenolic compounds. *Food Qual. Pref.* **2004**, *15*, 761–769.

(40) Asquith, T. N.; Uhlig, J.; Mehansho, H.; Putman, L.; Carlson, D. M.; Butler, L. Binding of condensed tannins to salivary proline-rich glycoproteins: The role of carbohydrate. *J. Agric. Food Chem.* **1987**, *35*, 331–334.

(41) Gambuti, A.; Rinaldi, A.; Pessina, R.; Moio, L. Evaluation of aglianico grape skin and seed polyphenol astringency by sds-page electrophoresis of salivary proteins after the binding reaction. *Food Chem.* **2006**, *97*, 614–620.

(42) Yan, Q.; Bennick, A. Identification of histatins as tannin-binding proteins in human saliva. *Biochem. J.* **1995**, *311*, 341–347.

(43) Rawel, H. M.; Frey, S. K.; Meidtner, K.; Kroll, J.; Schweigert, F. J. Determining the binding affinities of phenolic compounds to proteins by quenching of the intrinsic tryptophan fluorescence. *Mol. Nutr. Food Res.* **2006**, *50*, 705–713.

(44) Kallithraka, S.; Bakker, J.; Clifford, M. N. Evidence that salivary proteins are involved in astringency. *J. Sens. Stud.* **1998**, *13*, 29–43.

(45) Rossetti, D.; Yakubov, G. E.; Stokes, J. R.; Williamson, A. M.; Fuller, G. G. Interaction of human whole saliva and astringent dietary compounds investigated by interfacial shear rheology. *Food Hydrocolloids* **2008**, *22*, 1068–1078.

(46) Sarni-Manchado, P.; Cheynier, V.; Moutounet, M. Interactions of grape seed tannins with salivary proteins. *J. Agric. Food Chem.* **1999**, *47*, 42–47.

(47) Horne, J.; Hayes, J.; Lawless, H. T. Turbidity as a measure of salivary protein reactions with astringent substances. *Chem. Senses* **2002**, *27*, 653–659.

(48) Murray, N. J.; Williamson, M. P.; Lilley, T. H.; Haslam, E. Study of the interaction between salivary proline-rich proteins and a polyphenol by ^{1}h-nmr spectroscopy. *Eur. J. Biochem.* **1994**, *219*, 923–935.

(49) Pascal, C.; Poncet-Legrand, C.; Imberty, A.; Gautier, C.; Sarni-Manchado, P.; Cheynier, V.; Vernhet, A. Interactions between a non glycosylated human proline-rich protein and flavan-3-ols are affected by protein concentration and polyphenol/protein ratio. *J. Agric. Food Chem.* **2007**, *55*, 4895–4901.

(50) Canon, F.; Paté, F.; Meudec, E.; Marlin, T.; Cheynier, V.; Giuliani, A.; Sarni-Manchado, P. Characterization, stoichiometry, and stability of salivary protein-tannin complexes by esi-ms and esi-ms/ms. *Anal. Bioanal. Chem.* **2009**, 1–11.

(51) Kallithraka, S.; Bakker, J.; Clifford, M. N. Interaction of (+)-catechin, (-)-epicatechin, procyanidin b2 and procyanidin c1 with pooled human saliva *in vitro*. *J. Sci. Food Agric.* **2001**, *81*, 261–268.

(52) Soares, S. I.; Gonçalves, R. M.; Fernandes, I.; Mateus, N.; De Freitas, V. Mechanistic approach by which polysaccharides inhibit α-amylase/procyanidin aggregation. *J. Agric. Food Chem.* **2009**, *57*, 4352–4358.

(53) Kawamoto, H.; Nakatsubo, F.; Murakami, K. Quantitative determination of tannin and protein in the precipitates by high-performance liquid-chromatography. *Phytochemistry* **1995**, *40*, 1503–1505.

(54) Schägger, H.; Von Jagow, G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kda. *Anal. Biochem.* **1987**, *166*, 368–379.

(55) O'connell, K. L.; Stults, J. T. Identification of mouse liver proteins on two-dimensional electrophoresis gels by matrix-assisted laser desorption/ionization mass spectrometry of in situ enzymatic digests. *Electrophoresis* **1997**, *18*, 349–359.

(56) Zacharius, R. M.; Zell, T. E.; Morrison, J. H.; Woodlock, J. J. Glycoprotein staining following electrophoresis on acrylamide gels. *Anal. Biochem.* **1969**, *30*, 148–152.

(57) Vitorino, R.; Lobo, M. J. C.; Ferrer-Correira, A. J.; Dubin, J. R.; Tomer, K. B.; Domingues, P. M.; Amado, F. M. L. Identification of human whole saliva protein components using proteomics. *Proteomics* **2004**, *4*, 1109–1115.

(58) Castagnola, M.; Congiu, D.; Denotti, G.; Di Nunzio, A.; Fadda, M. B.; Melis, S.; Messana, I.; Misiti, F.; Murtas, R.; Olianas, A.; Piras, V.; Pittau, A.; Puddu, G. Determination of the human salivary peptides histatins 1, 3, 5 and statherin by high-performance liquid chromatography and by diode-array detection. *J. Chromatogr., B* **2001**, *751*, 153–160.

(59) Vitorino, R.; Barros, A.; Caseiro, A.; Domingues, P.; J., D.; Amado, F. Towards defining the whole salivary peptidome. *Proteomics Clin. Appl.* **2009**, *3*, 528–540.

(60) Beeley, J. A.; Sweeney, D.; Lindsay, J. C. B.; Buchanan, M. L.; Sarna, L.; Khoo, K. S. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of human parotid salivary proteins. *Electrophoresis* **1991**, *12*, 1032–1041.

(61) Pascal, C.; Bigey, F.; Ratomahenina, R.; Boze, H.; Moulin, G.; Sarni-Manchado, P. Overexpression and characterization of two human salivary proline rich proteins. *Protein Expression Purif.* **2006**, *47*, 524–532.

(62) Kirkland, T. N.; Finley, F.; Orsborn, K. I.; Galgiani, J. N. Evaluation of the proline-rich antigen of coccidioides immitis as a vaccine candidate in mice. *Infect. Immun.* **1998**, *66*, 3519–3522.

(63) De Freitas, V.; Mateus, N. Nephelometric study of salivary protein-tannin aggregates. *J. Sci. Food Agric.* **2002**, *82*, 113–119.

(64) Johansson, A.-K.; Lingström, 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.

(65) Brand, H.; Tjoe Fat, G.; Veerman, E. The effects of saliva on the erosive potential of three different wines. *Aust. Dent. J.* **2009**, *54*, 228–232.

(66) Bennick, A.; Mclaughlin, A. C.; Grey, A. A.; Madapallimattam, G. The location and nature of calcium-binding sites in salivary acidic proline-rich phosphoproteins. *J. Biol. Chem.* **1981**, *256*, 4741–4746.

(67) Hay, D. I.; Moreno, E. C.; Schlesinger, D. H. Phosphoprotein inhibitors of calcium phosphate precipitation from human salivary secretions. *Inorg. Perspect. Biol. Med.* **1979**, *2*, 271–285.

(68) Nayak, A.; Carpenter, G. H. A physiological model of tea-induced astringency. *Physiol. Behav.* **2008**, *95*, 290–294.

(69) Gillece-Castro, B. L.; Prakobphol, A.; Burlingame, A. L.; Leffler, H.; Fisher, S. J. Structure and bacterial receptor activity of a human salivary proline-rich glycoprotein. *J. Biol. Chem.* **1991**, *266*, 17358–17368.

(70) Hatton, M. N.; Loomis, R. E.; Levine, M. J.; Tabak, L. A. Masticatory lubrication. The role of carbohydrate in the lubricating property of a salivary glycoprotein-albumin complex. *Biochem. J.* **1985**, *230*, 817–820.

(71) 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.