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# Carbohydrates Inhibit Salivary Proteins Precipitation by Condensed Tannins

Susana Soares, Nuno Mateus, and Victor de Freitas*

Chemistry Investigation Center (CIQ), Department of Chemistry, Faculty of Sciences, University of Porto, Rua do Campo Alegre 687, 4169-007 Porto, Portugal

**ABSTRACT:** Condensed tannins are a group of polyphenols that are associated with the astringency sensation, as they readily interact and precipitate salivary proteins. As this interaction is affected by carbohydrates, the aim of this work was to study the effect of some carbohydrates used in the food industry [arabic gum (AG), pectin, and poligalacturonic acid (PGA)] on the salivary proteins/grape seed procyanidins interaction. This was assessed monitoring the salivary proteins that remain soluble in the presence of condensed tannins with the addition of carbohydrates (HPLC) and analysis of the respective precipitates (SDS-PAGE). The results show that pectin was the most efficient in inhibiting protein/tannin precipitation, followed by AG and PGA. The results suggest that pectin and PGA exert their effect by formation of a ternary complex protein/polyphenol/carbohydrate, while AG competes with proteins for tannin binding (competition mechanism). The results also point out that both hydrophilic and hydrophobic interactions are important for the carbohydrate effects.

**KEYWORDS:** *proline-rich proteins, grape seed tannins, pectin, arabic gum, polygalacturonic acid*

## INTRODUCTION

Condensed tannins are a complex group of polyphenolic polymers of catechin (polyphenol compounds) that can be found in vegetal foodstuffs, particularly in fruits, cereal grains, and beverages (red wine, tea, and beer). During foodstuff consumption, these polyphenols interact with salivary proteins forming insoluble aggregates that are supposed to be at the origin of the astringency sensation.[1−4] Astringency has been defined as a dryness of the oral surface, puckering and tightening sensations of the oral mucosa typically experienced during the ingestion of tannin-rich food, in particular red wine.[5] It is considered to be a tactile, diffuse, and poorly localized sensation. In fact, this sensation is often the last one to be detected as it can take 15 s or more for the perception to fully develop.[6]

Although there are a few theories about the origin of the astringency sensation, the most widely accepted one relies on the interaction between tannins and salivary proteins. In general, these tannin−protein interactions are thought to involve the cross-linking of separate protein molecules by the tannin, which acts as a polydentate ligand on the protein surface involving hydrophobic and hydrogen bonds.[7−12]

Salivary proteins include very structurally diverse proteins such as $\alpha$-amylase, albumin, lysozyme, proline-rich proteins (PRPs), histatins, cystatins, and statherin. The main salivary proteins have been grouped into six structurally related major classes, namely, histatins, basic proline-rich proteins (bPRPs), acidic proline-rich proteins (aPRPs), glycosylated proline-rich proteins (gPRPs), statherin, and cystatins.[13,14] These proteins have important biological functions in saliva associated with calcium binding to enamel, maintenance of ionic calcium concentration (PRPs and statherin), antimicrobial action (histatins and cystatins), or protection of oral tissues against degradation by proteolytic activity (cystatins).[15−19]

Regarding tannin−protein interactions in vitro, these are reported to be affected by several factors, in particular ionic strength, pH of the medium, percentage of ethanol, temperature, and presence of carbohydrates.[20−24]

The first report of the inhibitory effect of carbohydrates in those interactions concerned the proposed mechanisms for the astringency loss during fruit ripening: as the cellular structure softens during fruit ripening, there is an increase in water-soluble pectin fragments that could prevent the formation of aggregates between fruit tannins and salivary proteins in the mouth, leading to a modified astringency response.[25−27] Other studies showed that the astringency of tannins, as well as their interaction with proteins, is reduced by the addition of carbohydrates.[23,28,29]

Two mechanisms have been proposed to explain this inhibitory effect of carbohydrates: (I) carbohydrates form ternary complex protein−polyphenol−carbohydrates, which enhance solubility in an aqueous medium; (II) there is a molecular association in solution between carbohydrates and polyphenols hence competing for protein aggregation.[25,27]

The effect of carbohydrates in protein−tannin interactions has a great impact in the perception and choice of foodstuffs. Carbohydrates are frequently used in food industry as food colloids (gums) and are also naturally present in several food products, thereby affecting their astringent features.

Despite the knowledge about the effect of carbohydrates on protein−tannin interactions, there is still little information when considering this effect on the interaction of tannins with salivary proteins. In fact, there is a lack of information in which way the structure of the different salivary proteins and the

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different carbohydrates affect their action. Most of the literature on this subject has mainly reported studies involving other proteins, namely, model proteins such as bovine serum albumin (BSA) and $\alpha$-amylase.

Therefore, this work aimed to study the effect of several carbohydrates used in the food industry as food colloids [arabic gum (AG), pectin, and polygalacturonic acid (PGA)] on the interaction between salivary proteins and grape seed fraction (GSF) (condensed tannins).

## MATERIALS AND METHODS

**Reagents.** All reagents used were of analytical grade. AG (purity not provided) was purchased from Aldrich. Pectin (purity 79.5%) and PGA (purity 80%) (both from citrus peel) were purchased from Sigma.

**GSF Isolation.** Procyanidins were extracted from grape seeds (*Vitis vinifera*) with an ethanol/water/chloroform solution (1:1:2, v/v/v). The resulting solution was centrifuged, and the chloroform phase, containing chlorophylls, lipids, and other undesirable compounds, was rejected. The hydroalcoholic phase was then extracted with ethyl acetate, and the organic phase was evaporated using a rotary evaporator (30 °C). The resulting residue corresponding essentially to oligomeric procyanidins was fractionated through a TSK Toyopearl HW-40(s) gel column (100 mm × 10 mm i.d., with 0.8 mL $min^{-1}$ methanol as eluent), yielding two fractions according to the method described in the literature.[30] The first fraction was obtained after elution with 99.8% (v/v) methanol during 5 h (240 mL), and the second was eluted with methanol/5% (v/v) acetic acid during the next 14 h (670 mL). Both fractions were mixed with deionized water, and the organic 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 electrospray ionization mass spectrometry (ESI-MS) (Finnigan DECA XP PLUS) as described in the literature.[31] The first fraction contains mainly catechins, procyanidin dimers, and their galloyl derivatives, and the second fraction contains essentially procyanidin dimers galloylated, procyanidin trimers and their galloyl derivatives, and procyanidin tetramers. For the second fraction, the average full mass spectra was obtained, and the mean degree of polymerization (mDP) was estimated from the ratio between the sum of the relative abundance of each compound multiplied by its number of elementary catechin units and the sum of the relative abundances for all compounds in the fraction. The fraction has a mean MW of 936 and a polymerization degree average of 3.2. Only the second fraction named GSF was used herein, as it was shown to be the most reactive toward proteins.

**Saliva Collection.** Saliva was collected from six healthy non-smoking volunteers, and 2 mL of saliva from each volunteer was used to make a saliva pool (whole saliva). The collection time was standardized at 2 p.m. to reduce concentration variability connected to circadian rhythms of secretion.[32] The saliva pool was mixed with 10% trifluoroacetic acid (TFA) (final concentration, 0.1%) to precipitate several high molecular weight salivary proteins (such as $\alpha$-amylase, mucins, carbonic anhydrase, and lactoferrin) and to preserve sample protein composition, since TFA partially inhibits intrinsic protease activity. After the centrifugation (8000*g* for 5 min), the supernatant (acidic saliva, AS) was separated from the precipitate and used for the following experiments.

**Pectin Purification.** Pectin was purified by precipitation with ethanol. Briefly, after dissolution in a small amount of water, ethanol was added to achieve a 70% (v/v) $CH_3CH_2OH/H_2O$ concentration. The precipitated carbohydrate was recovered by filtration under vacuum (1 $\mu$m filters) and dried. Pectin was analyzed by colorimetric methods and gas chromatography to determine sugar composition and esterification degrees.[33,34] It was found to be composed by 85% galacturonic acid, 10% galactose, and 5% other sugars. The degrees of methylation and acetylation were determined to be 14 and 1%, respectively.

**Protein and Tannin Interaction.** The AS sample was analyzed by high-performance liquid chromatography (HPLC) before and after the interaction with increasing concentrations of GSF. These experiments were made to obtain the minimal GSF concentration that precipitates almost totally the salivary proteins. The control condition was a mixture of AS (150 $\mu$L) and water (50 $\mu$L) (final volume, 200 $\mu$L). Different volumes of a GSF stock solution (30.0 mM) prepared in water were added to AS (150 $\mu$L) to obtain the desired final concentrations, 150, 300, or 750 $\mu$M. The final volume was adjusted to 200 $\mu$L with pure water. The mixture was shaken and kept for 5 min at room temperature (±20 °C) and then centrifuged (8000*g*, 5 min). The supernatant was injected into the HPLC. After these first experiments, the GSF concentration chosen for the experiments with the carbohydrates was 300 $\mu$M.

**Effect of Carbohydrates on Protein/Tannin Interaction.** For the experiments with carbohydrates, stock solutions of AG (25.0 g $L^{-1}$), PGA (30.0 g $L^{-1}$), and pectin (10.0 g $L^{-1}$) were prepared in water, and different volumes of these stock solutions were added to GSF solution to obtain different final concentrations. The final volume (50 $\mu$L) was adjusted with water to obtain the desired carbohydrates final concentration (between 2.4 and 30.0 g $L^{-1}$, depending on carbohydrates). The mixture was shaken and kept at room temperature (±20 °C) for 30 min. After that, 150 $\mu$L of AS was added to the previous mixture (final volume, 200 $\mu$L), which was shaken and kept for 5 min at room temperature (±20 °C). The mixture was then centrifuged (8000*g*, 5 min), the supernatant was injected into the HPLC, and the precipitate was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). The pH of the supernatant was measured prior to analysis, and it was 3.1 [which is near to some beverages pH such as red wine (pH 3.5)].

**HPLC Analysis.** Ninety microliters of each solution was injected on a HPLC Lachrom system (L-7100) (Merck Hitachi) equipped with a Vydac C8 column (Grace Davison Discovery Sciences), with 5 $\mu$m particle diameter (column dimensions 150 mm × 2.1 mm); detection was carried out at 214 nm, using a UV−vis detector (L-7420). The HPLC solvents were 0.2% aqueous TFA (eluent A) and 0.2% TFA in acetonitrile (ACN)/water 80/20 (v/v) (eluent B). The gradient applied was linear from 10 to 40% (eluent B) in 60 min, at a flow rate of 0.30 mL $min^{-1}$. After this program, the column was washed with 100% eluent B for 20 min to elute S type cystatins and other late-eluting proteins. After washing, the column was stabilized with the initial conditions.[1,32]

**SDS-PAGE.** The precipitates that resulted from the interaction between AS and GSF in absence and presence of the highest carbohydrates concentration, as well as the AS control solution, were analyzed by SDS-PAGE. The precipitates were resolubilized in 200 $\mu$L of electrophoresis sample buffer (50 mM Tris-HCl, pH 6.8, 12% v/v glycerol, 4% SDS, 2.5% v/v $\beta$-mercaptoethanol, and 0.01% bromophenol blue) to have the same initial volume used in the previous experiments during precipitation. Eighty microliters of electrophoresis sample buffer was added to 80 $\mu$L of these solutions. The control solution was composed by 80 $\mu$L of the AS solution and 80 $\mu$L of a twice-concentrated electrophoresis sample buffer (100 mM Tris-HCl, pH 6.8, 24% v/v glycerol, 8% SDS, 5% v/v $\beta$-mercaptoethanol, and 0.02% bromophenol blue). The samples were heated at 60 °C for 1 h with shaking and then analyzed by SDS-PAGE in a tris-tricine buffer system according to the method of Schägger using 16% acrylamide resolving gel. The stacking gel was 5% acrylamide. The cathode buffer was 0.1 M Tris, 0.1 M tricine, and 0.1% SDS. 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) at constant voltage (150 V). After electrophoresis, the gels were stained with Imperial Protein Stain (Thermo Scientific), a Coomassie R-250 dye-based reagent. 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 (Sigma) were estimated by comparison with the migration rates of standard proteins ($\beta$-galactosidase from *Escherichia coli*, 116000 Da; phosphorylase b from rabbit muscle, 97000 Da; albumin bovine serum, 66000

Da; glutamic dehydrogenase from bovine liver, 55000 Da; ovalbumin from chicken egg, 45000 Da; glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle, 36000 Da; carbonic anhydrase from bovine erythrocytes, 29000 Da; trypsinogen from bovine pancreas, 24000 Da; trypsin inhibitor from soybean, 20000 Da; $\alpha$-lactalbumin from bovine milk, 14200 Da; and aprotinin from bovine lung, 6500 Da).

**Half Maximal Effective Concentration ($EC_{50}$) Calculation and Statistical Analysis.** $EC_{50}$ values were calculated considering the area variation of chromatogram peaks of each protein in the presence of carbohydrates. The software GraphPad Prism 5 (GraphPad Software, Inc.) was used to calculate $EC_{50}$ and statistical analysis. Statistical significance of the difference between the several calculated $EC_{50}$ was evaluated by one-way analysis of variance, followed by the Bonferroni test. Differences were considered to be statistically significant when $P < 0.05$. All of the experiments were performed in $n = 3$ repetitions.

## RESULTS AND DISCUSSION

The carbohydrates AG, pectin, and PGA are commonly present in food and are also used in the food industry as additives. The influence of these three different ionic carbohydrates on the interaction between salivary proteins and grape seed condensed tannins was assessed by HPLC analysis and SDS-PAGE. The disrupting effect of these compounds on protein−tannin aggregates was assessed by monitoring the increase of the salivary proteins that remain soluble in the presence of condensed tannins with the addition of each of these carbohydrates.

**Salivary Proteins.** The initial acidic treatment of human saliva with TFA is used to precipitate several high molecular weight salivary proteins (such as $\alpha$-amylases, mucins, carbonic anhydrase, and lactoferrin) and to preserve sample protein composition, since TFA partially inhibits intrinsic protease activity.[32] However, peptides and proteins like histatins, basic, acidic, and glycosylated PRPs, statherin, cystatins, and defensins are soluble in AS solution and may be directly analyzed by RP-HPLC, as previously described.[1,32]

The HPLC chromatogram of this AS solution at 214 nm is presented in Figure 1. The top of the figure shows the distribution of the different families of salivary proteins along the chromatogram that were established previously by proteomic approaches, namely, ESI-MS and matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF).[1,32]

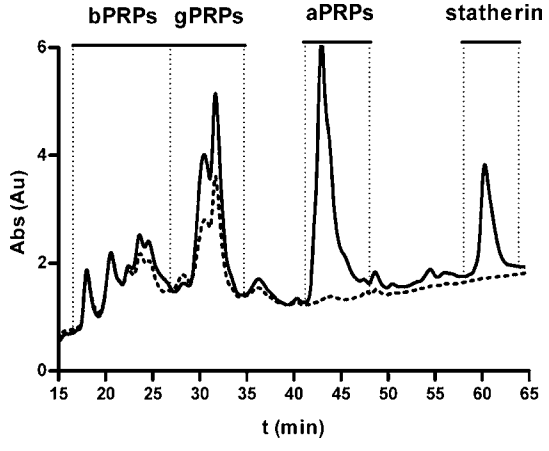


**Figure 1.** Typical RP-HPLC profile detected at 214 nm of the AS solution of human saliva in the absence (—) and presence (- - -) of 300 $\mu$M GSF. The vertical dotted lines show the ranges and the main salivary proteins family assigned to each HPLC peptide region: bPRPs, gPRPs, and aPRPs.

The HPLC chromatogram of the AS solution is roughly divided into four salivary proteins family regions: the first region comprises proteins that belong to the classes of bPRPs and histatins. The bPRPs identified in this region include IB-8b, IB-8c, IB-9, IB-4, and P-J, and the histatins include histatins 3, 5, 7, 8, and 9. The second region comprises mainly one gPRPs, the bPRP3. The next region corresponds entirely to aPRPs, namely, PRP1 and PRP3, and the last region has phosphorylated and nonphosphorylated forms of statherin and peptide P-B.[1]

**Interaction of Salivary Proteins with Procyanidin Fraction from Grape Seeds (GSF).** The first experiments were made to assess the minimal procyanidin concentration that leads to a significant precipitation of salivary proteins and then to study if the carbohydrates were able to inhibit that precipitation. It was important to establish this concentration since an insufficient quantity of procyanidins would lead to no significant precipitation of most of the salivary proteins, and an excess of GSF would lead to free molecules of GSF, thereby influencing the further effect of carbohydrates. Bearing this, three increasing GSF concentrations were tested (150, 300, and 750 $\mu$M).

From these experiments, the GSF concentration chosen for the experiments with carbohydrates was 300 $\mu$M, since it corresponded to the GSF concentration that precipitated almost all of the salivary proteins present in the AS (Figure 1). In fact, while for 150 $\mu$M GSF only aPRPs and statherin were significantly affected, the 750 $\mu$M concentration led to the precipitation of all of the proteins but also to an excess of free molecules of GSF (data not shown).

It can be seen from Figure 1 that the addition of 300 $\mu$M GSF reduced significantly the amount of gPRPs and practically depleted aPRPs and statherin. On the other hand, bPRPs were not significantly affected. Therefore, the effect of carbohydrates on the interaction between salivary proteins and tannins was focused on gPRPs, aPRPs, and statherin proteins.

**Effect of Carbohydrates on the Interaction between Salivary Proteins and GSF.** The experimental approach described herein intends to mimic the phenomenon that occurs in the mouth during food mastication, where tannins and carbohydrates simultaneously contact with salivary proteins. So, a solution of tannins−carbohydrates was prepared, to which the AS solution was subsequently added. The control experimental condition was made only with AS and 300 $\mu$M of GSF. Figure 2 shows part of the chromatogram corresponding only to the families of salivary proteins that were effectively affected by the GSF in the absence or presence of the different carbohydrates.

From the presented results, it can be observed that the carbohydrates in solution enhance the chromatographic peaks corresponding to salivary proteins, in especially for aPRPs and statherin. Figure 3 shows variation of the chromatographic peaks area of the several salivary proteins studied with the increase in the carbohydrate concentration, expressed in percentage of the area of these proteins relatively to the respective area in control saliva (AS without tannin).

In general, it is possible to observe that salivary proteins in solution increase concomitantly with the carbohydrates concentration. The observed changes may be interpreted as the inhibition of salivary proteins precipitation by tannins in the presence of increasing concentrations of carbohydrates.

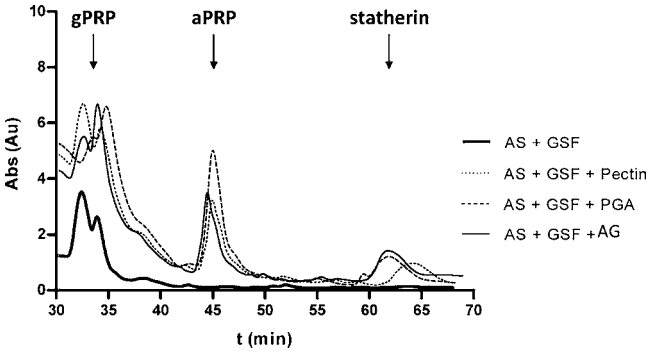


**Figure 2.** Part of the chromatograms of the AS solution after the interaction with GSF (300 $\mu$M) in the absence (AS solution + 300 $\mu$M GSF) and presence of the several tested carbohydrates (pectin, 5.0 g $L^{-1}$; AG, 10.0 g $L^{-1}$; and PGA, 20.0 g $L^{-1}$).

Pectin was by far the most effective carbohydrate in preventing precipitation of salivary proteins by condensed tannins. While pectin prevents almost total precipitation of salivary proteins at the concentration of 10.0 g $L^{-1}$, AG and PGA only showed similar effects for concentrations higher than 20.0 g $L^{-1}$.

Besides the HPLC analysis of the supernatant, it was also important to analyze the pellets that resulted from the AS interaction with GSF in the absence and presence of the different carbohydrates. So, to analyze these pellets, all precipitates were resolubilized by heating (60 °C) in 200 $\mu$L of electrophoresis sample buffer and further analysis by SDS-PAGE. The proteins bands were subsequently analyzed by densitometry, and the results are presented in Figure 4.

The results obtained by SDS-PAGE analysis of the referred pellets, as well as by the densitometry analysis performed to the gel, clearly demonstrate that the presence of these carbohydrates, in particular pectin and AG, decreases the salivary proteins that are precipitated by tannins (Figure 4). The maximum of salivary proteins precipitation results from the interaction between AS and GSF in the absence of AG and pectin (lane C). The experiment with AS and GSF resulted in a value of 0.46, while in the presence of pectin or AG, the obtained value is significantly lower (0.16 and 0.11, respectively). Regarding the PGA effect, looking at the SDS-PAGE results, it is also possible to observe that it also leads to a decrease of salivary proteins precipitation but in a fewer extent than the other carbohydrates. SDS-PAGE analyses are probably not comparable with HPLC results because there are other proteins (cystatins and other late eluting proteins) not analyzed by HPLC that could be precipitated by tannins and so appear in the SDS-PAGE gel. In this way, SDS-PAGE results only reflect the efficiency of the carbohydrates in inhibiting tannin−protein interaction.

To compare properly the effectiveness of carbohydrates to inhibit salivary proteins precipitation by tannins, the half maximal effective concentration ($EC_{50}$) was calculated from the HPLC results (Table 1). Pectin has been shown to have the lowest $EC_{50}$ value (around 4−5 g $L^{-1}$) being the most effective in the inhibition of salivary proteins precipitation by tannins, while PGA was shown to be the less effective carbohydrate in inhibiting tannin−salivary proteins precipitation with an $EC_{50}$ between 14 and 19 g $L^{-1}$.

Oppositely to AG, the effect of the other carbohydrates is quite similar for the three groups of salivary proteins, especially

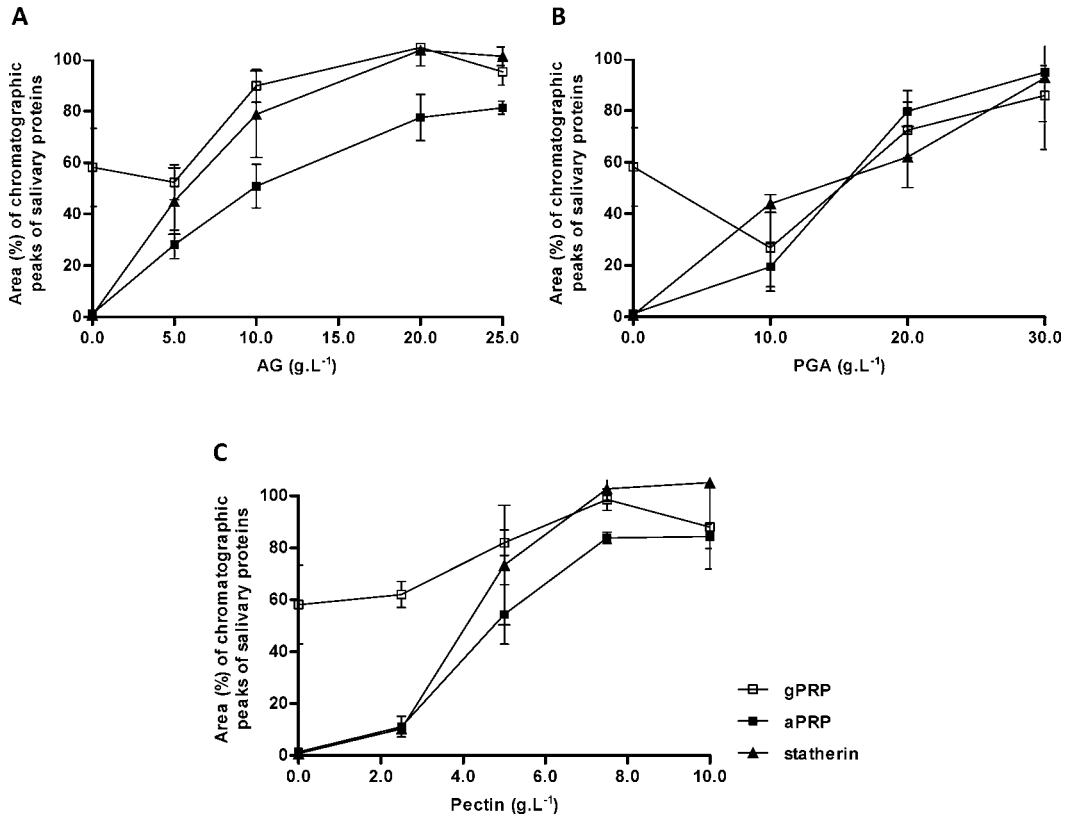


**Figure 3.** Influence of carbohydrate concentration on salivary proteins precipitation by condensed tannins (GSF, 300 $\mu$M). (A) AG, (B) PGA, and (C) pectin. These results represent the average of three independent experiments.


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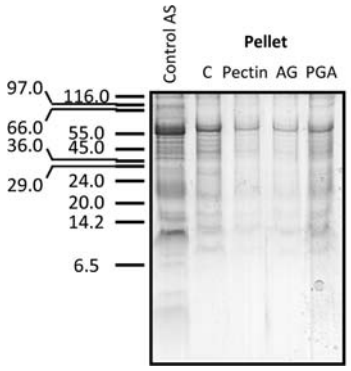


| | Ratio |
|---|---|
| Control AS | 1.00 ± 0.07 |
| C (AS + GSF) | 0.46 ± 0.00[a] |
| Pectin | 0.16 ± 0.02[b] |
| AG | 0.11 ± 0.02[b] |
| PGA | 0.43 ± 0.08[a] |

**Figure 4.** SDS-PAGE of the pellets that resulted from the interaction between AS and GSF in the absence (C) and presence of the several carbohydrates (pectin, 10.0 g $L^{-1}$; AG, 25.0 g $L^{-1}$; and PGA, 25.0 g $L^{-1}$). The molecular weight markers were substituted by lines, and the molecular mass marked on the left side is expressed in kDa. The gels were stained with Imperial Protein Stain, a Coomassie R-250 dye-based reagent. The table shows the ratio between the densitometry values obtained for total salivary proteins present in AS (control AS) and in each experiment. The values with equal letters are not significantly different ($P < 0.05$).

**Table 1. Half-Maximal Effective Concentration ($EC_{50}$) in Inhibition of Tannin/Salivary Protein Precipitation by Three Carbohydrates: AG, PGA, and Pectin[a]**

| | $EC_{50}$ (g $L^{-1}$) | | |
|---|---|---|---|
| carbohydrate | gPRP | aPRP | statherin |
| AG | 4.78 ± 0.39 a | 10.10 ± 0.16 | 5.68 ± 0.65 a |
| PGA | 19.47 ± 0.50 | 15.12 ± 0.58 f | 14.34 ± 1.61 f |
| pectin | 4.75 ± 0.38 d | 4.83 ± 0.10 d | 4.77 ± 0.18 d |

[a]The values with equal letters are not significantly different ($P < 0.05$).

for pectin, which indicates that the influence at this latter is independent of the salivary protein structure. For AG, its effect is more pronounced for gPRPs and statherin rather than for aPRPs. In fact, the $EC_{50}$ obtained for AG and aPRP is nearly twice of the one obtained for the other salivary proteins. These results seem to suggest that the mechanism by which AG exerts its effect is probably different and more selective toward salivary protein structure as compared to pectin and PGA.

It had already been shown that AG inhibits the interaction between a protein ($\alpha$-amylase) and condensed tannins by a different mechanism than pectin.[23] While pectin was described to have the ability to form a ternary complex protein–polyphenol–carbohydrate (Figure 5, i), thereby enhancing its solubility in aqueous medium, resulting in less insoluble aggregates, AG has been described to have the ability to inhibit protein–tannin interaction by competing with tannins by protein aggregation (Figure 5, ii). Although this previous work was performed with a different protein ($\alpha$-amylase),[23] a similar behavior could explain the high efficiency and lack of selectivity observed herein for pectin and PGA. In the present case, as tannins complex with both proteins and carbohydrates (pectin or PGA), there is no competition mechanism and thus less selectivity.

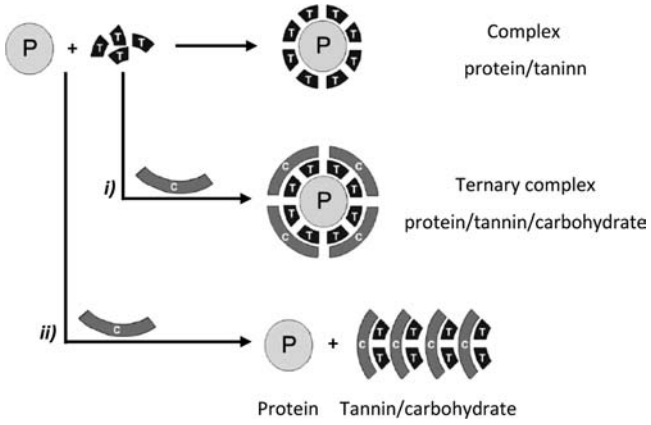


**Figure 5.** Possible mechanism (i and ii) involved in the inhibition of the aggregation of tannins and proteins by carbohydrates. P, protein; T, tannin; and C, carbohydrate.[40]

PGA and pectin are quite similar polymers of $\alpha$-(1→4)-linked D-galacturonic acid units that differ essentially on the degree of methoxylation of their carboxyl groups.[35] This structural similarity probably explains the identical inhibition mechanism described, leading to the formation of ternary complexes. However, pectin is more esterified than PGA, which reduces its polarity and consequently its readiness to hydrophilic interactions. On the other hand, esterification favors its ability to establish van der Waals interactions. Thus, pectin would be more prone to hydrophobic interactions than PGA, which favors the interaction with condensed tannins (that tend to be more hydrophobic molecules). This particular feature seems to be important to increase the ability of pectin to inhibit salivary protein–tannin precipitation despite its low selectivity.

Regarding AG, its structure is quite different from the structures of pectin and PGA, which could explain the different mechanism of inhibition of this carbohydrate. A competition mechanism is once again proposed herein for AG (Figure 5, ii). The effectiveness of this process is expected to depend on the relative affinities of tannin toward AG and salivary proteins. If tannins have a higher affinity to AG than to salivary proteins, AG would be able to prevent salivary proteins–tannins precipitation. AG is a heteropolysaccharide composed of a polysaccharide and hydroxyproline-rich protein moieties able to ensure hydrophobic interactions with procyanidins. The polysaccharide moiety has also a slightly acidic character, resulting from the presence of 20% glucuronic acids. The acidic character may help to establish electrostatic and hydrogen bonds that could help to strengthen the interaction with condensed tannins. On the other hand, the presence of the protein–proline moiety could also contribute to an increase in the efficiency of AG to complex strongly with tannins, competing for the interaction with salivary proteins. This is


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readily apparent from the obtained results as AG was found to be the second more efficient carbohydrate in a range of concentrations generally much lower than the ones for PGA.

Altogether, these results seem to point out that both hydrophilic and hydrophobic interactions are important for the carbohydrate effects where the hydrophilic interactions (hydrogen bonds) seem to contribute to the selectivity of the inhibition, while the hydrophobic ones seem to contribute to the efficiency. This was especially clear for pectin that was shown to be the most efficient in inhibiting salivary proteins precipitation by condensed tannins toward the aPRP, gPRP, and statherin, despite its low selectivity.

Besides the central role of the carbohydrate structure, these results also point to the importance of the protein structure for the effect of those carbohydrates. PRPs are classified in different classes (acidic, basic, and glycosylated) based on small and specific structural differences, in particular richness in acidic or basic residues and the presence of sugar molecules in their structure.[36−38] The acidic character of aPRPs is confined roughly to the first 30 amino acids, due to the presence of many aspartic and glutamic acid residues. The remaining part is basic and, similarly to bPRPs, shows repeated sequences of proline and glutamine often separated by glycine residues. Regarding the gPRP, there is little information about the glycosylation pattern of these proteins. Statherin is abundant in tyrosine residues and is phosphorylated at Ser-2 and Ser-3. This latter protein also has a highly acidic amino-terminal hexapeptide (first six residues) that is needed to inhibit calcium phosphate crystal growth. These structural aspects of salivary proteins are important for their competition with carbohydrates toward tannins in the competition mechanism previously referred. In fact, the efficiency of the carbohydrate to inhibit protein−tannin aggregation depends not only on the carbohydrate structure but also on the affinity between the salivary proteins and the tannins. This is evident for AG that has been shown to be less efficient (high $EC_{50}$) in inhibiting aPRPs−tannin aggregation as compared to other salivary proteins. In this case, AG has been shown to be more effective in inhibiting gPRPs−tannins and statherin−tannins precipitation ($EC_{50}$ = 4.78 and 5.68 g $L^{-1}$, respectively) than aPRPs−tannin precipitation ($EC_{50}$ = 10.10 g $L^{-1}$). In fact, aPRPs have been described recently to interact more strongly with condensed tannins than the other salivary protein families.[1] On the other hand, gPRPs are known to not interact strongly with tannins as compared to other salivary proteins because of the presence of the sugar moiety in their structure,[39] which probably explains the higher inefficacy of AG to inhibit gPRP−tannin precipitation.

Overall, the obtained results show that carbohydrates commonly used in the food industry are able to inhibit the interaction and precipitation of salivary proteins with tannins and thus influence the perceived astringency of some food products. The extent and the mechanism by which this inhibition occurs are related with the carbohydrate structure and in the last instance also to the protein structure.

## AUTHOR INFORMATION

### Corresponding Author

*Tel: +351 220402558. Fax: +351 220402659. E-mail: vfreitas@fc.up.pt.

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### Notes

The authors declare no competing financial interest.

## ABBREVIATIONS USED

ACN, acetonitrile; aPRPs, acidic proline-rich proteins; AS, acidic saliva; AG, arabic gum; bPRPs, basic proline-rich proteins; ESI-MS, electrospray ionization mass spectrometry; gPRPs, glycosylated proline-rich proteins; GSF, grape seed fraction; HPLC, high-performance liquid chromatography; MALDI-TOF/TOF, matrix-assisted laser desorption/ionization time-of-flight/time-of-flight; PGA, polygalacturonic acid; PRPs, proline-rich proteins; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid

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