DOI:10.1021/jf900302r

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

# Mechanistic Approach by Which Polysaccharides Inhibit α-Amylase/Procyanidin Aggregation

Susana I. Soares, Rui M. Gonçalves, Iva Fernandes, Nuno Mateus, and Victor de Freitas*

Chemistry Investigation Centre, Department of Chemistry, Faculty of Sciences, University of Porto, Rua do Campo Alegre 687, 4169-007 Porto, Portugal

The present work studies the inhibition of aggregation of α-amylase and procyanidin fractions by different polysaccharides (arabic gum, β-cyclodextrin, and pectins). Several analytical approaches, namely, fluorescence quenching, nephelometry, and dynamic light scattering (DLS), were used. In general, nephelometry showed that the presence of the polysaccharides in solution reduced the formation of insoluble aggregates. The fluorescence quenching measurements showed two effects: arabic gum and β-cyclodextrin reduce the quenching effect of procyanidin fractions on α-amylase fluorescence, whereas pectins do not affect the quenching of α-amylase fluorescence by procyanidin fractions. DLS measurements have revealed that the polysaccharides studied induce a decrease in aggregates size, which probably is due to the formation of smaller aggregates resulting from the disruption and reorganization of the procyanidin fractions/α-amylase aggregates. Overall, the results obtained for arabic gum and β-cyclodextrin strongly suggest that the main mechanism by which these two compounds inhibit protein/polyphenol aggregation is by molecular association between these polysaccharides and polyphenols, competing with protein aggregation. In the case of pectins, the results obtained provide evidence that the main mechanism by which they reduce protein/polyphenol aggregation is by forming a protein/polyphenol/polysaccharide complex, enhancing its solubility in aqueous medium.

**KEYWORDS: Aggregation; α-amylase; fluorescence; polysaccharides; tannin**

## INTRODUCTION

Tannins are phenolic compounds, yielded from the secondary metabolism of higher plants, being found worldwide in many different families of plants (*1*). Bate-Smith and Swain defined the plant tannins as water-soluble phenolic compounds with molecular masses between 300 and 3000 Da, displaying the usual phenolic reactions [e.g., blue color with iron(III) chloride] and precipitating alkaloids, gelatins, and other proteins (*2*, *3*). However, this definition does not include all tannins. Indeed, more recently, molecules with a molecular mass of up to 20000 Da have been isolated and should also be classified as tannins on the basis of their phenolic reactions. High concentrations of tannins can be found in nearly every part of the plant, such as in wood, leaves, fruit, roots, and seed. Usually, tannins are divided in two major classes: condensed (proanthocyanidins) and hydrolyzable tannins. The first ones are polymers of catechin, and the latter are gallic or ellagic esters of glucose. It is assumed that these compounds have a biological role in the plant related to protection against infection and herbivores (*1*).

From a nutritional point of view, the interaction between tannins and enzymes, such as α-amylase or trypsin, has been shown to have harmful effects, with the inhibition of these enzymes and decrease in body weight gain (*4*−*6*).

On the other hand, the interaction of α-amylase and other salivary proteins such as proline-rich proteins (PRPs) and histatins with tannins is thought to be responsible for the astringency sensation. This event is thought to result from the formation of protein/tannin insoluble aggregates that precipitate, reducing the palate lubrication and causing an unpleasant sensation of roughness, dryness, and constriction (*7*, *8*). Astringency is often perceived as a negative attribute as in dairy products, nuts, and juices (*9*). However, in some beverages such as tea, beer, and red wine, astringency could be perceived as a positive quality factor, if not too intense.

The affinities of salivary proteins to complex tannins depend on many factors and mainly on their chemical structures (*10*, *11*). Previous works have shown that a PRP (IB8c) binds to condensed tannins much more effectively than α-amylase (*12*). This can be explained not only by their primary structure but also by the three-dimensional structure of these proteins. In fact, whereas α-amylase is a globular protein, PRPs are extended randomly coiled proteins, which offer more sites to interact with tannins. However, α-amylase seems to be more specific and selective than PRPs in the aggregation with samples containing different amounts of proanthocyanidins (*13*). α-Amylase from porcine pancreas (hereafter PPA) is a single polypeptide chain of 496 residues of

*Corresponding author (e-mail vfreitas@fc.up.pt; telephone +351226082858; fax +351226082959).

 Published on Web 4/20/2009 

which 17 are tryptophan residues, which are responsible for its intrinsic fluorescence (*14*). Mammalian α-amylases show a high degree of homology, so it is considered that in a general way porcine and human α-amylases are very similar (*15*, *16*).

The presence of polysaccharides in solution is also an important factor that affects the interaction between tannins and proteins. Several studies have demonstrated the ability of some neutral and anionic polysaccharides to disrupt the binding of polyphenols to proteins (*17*−*19*). For instance, loss of astringency is one of the principal changes that occur during the ripening of many edible fruits, which has been related to the increase of soluble pectins during maturation. Two mechanisms have been proposed to explain this phenomenon: (I) polysaccharides form a ternary complex protein/polyphenol/polysaccharide, which enhances solubility in an aqueous medium; (II) there is a molecular association in solution between polysaccharides and polyphenols, competing for protein aggregation (*17*, *19*).

The effect of polysaccharides on polyphenol complexation with proteins has been studied in solution by techniques such as enzyme activity, NMR, and nephelometry (*17*, *19*, *20*). Whereas NMR gives important data about the interaction between tannin and protein at a molecular level, nephelometry measures the formation of high molecular weight structures at a macromolecular level, taking into account all physical−chemical driving forces involved in the aggregates' formation. Over recent years, the fluorescence quenching technique has been employed, with success, to study the specificity of the protein/tannin interaction at a molecular level (*21*−*23*). Changes on PPA structure resulting from the interaction with tannins were evaluated by the measurement of intrinsic fluorescence intensity of protein tryptophan residues. Fluorescence measurements give information about the molecular environment in the vicinity of the chromophore molecule. The decrease of protein intrinsic fluorescence intensity is called quenching, which can occur by different mechanisms, namely, collisional quenching, when the excited-state fluorophore is deactivated upon contact with some other molecule in solution (the quencher), or static quenching, whereby fluorophores form nonfluorescent complexes with quenchers (*24*).

The aim of this work is to link the fluorescence quenching, nephelometry, and dynamic light scattering (DLS) techniques to provide insights, at a molecular level, of the process whereby polysaccharides (arabic gum, β-cyclodextrin, and pectins with different methoxylation degrees) inhibit α-amylase aggregation with grape seed procyanidins. α-Amylase/tannin aggregates are thought to influence food astringency and, on the other hand, inhibit enzyme activity.

## MATERIALS AND METHODS

**Reagents.** α-Amylase from porcine pancreas (≥98%) was purchased from Sigma. Arabic gum was purchased from Aldrich. β-Cyclodextrin (≥99%, HPLC) was purchased from Fluka. Pectins (from citrus peel and standardized by the addition of sucrose) with different methoxylation degrees (MD of 27, 58−63, and 72%) were kindly supplied by CPKelco Co.

**Grape Seed Tannin Isolation.** Condensed tannins were extracted as described in the literature (*25*, *26*). Briefly, condensed tannins were extracted from *Vitis vinifera* grape seeds with an ethanol/water/chloroform solution (1:1:2, v/v/v), and the chloroform phase, containing chlorophylls and lipids, was rejected. The resulting hydroalcoholic phase was extracted with ethyl acetate. The organic solvent was removed using a rotary evaporator (30 °C), and the resulting residue, corresponding to catechin monomers and oligomeric procyanidins, was fractionated through a TSK Toyopearl gel column TSK Toyopearl HW-40(s) gel column (100 mm × 10 mm i.d., with 0.8 mL $min^{-1}$ methanol as eluent), yielding four fractions. Fractions I and II were obtained after elution with 99.8% (v/v) methanol during 1 and 5 h, respectively (cumulative time); fraction III was obtained after elution with methanol/5% (v/v) acetic acid during the next 14 h; and fraction IV was obtained after elution with methanol/10% (v/v) acetic acid during the next 8 h. All of the fractions were mixed with deionized water; the solvent was eliminated using a rotary evaporator under reduced pressure at 30 °C and then freeze-dried. The composition of procyanidins in each fraction was determined by direct analysis by ESI−mass spectrometry (Finnigan DECA XP PLUS) as described in the literature (*26*). Fraction I contains catechin, galloyl derivatives, procyanidin dimer, the galloyl derivative, and procyanidin trimer; fraction II contains procyanidin trimer and tetramer and its galloyl derivatives (mean MW = 950); fraction III contains procyanidin pentamer and the galloyl derivative (mean MW = 1512); fraction IV contains procyanidin pentamer digalloyl, procyanidin tetramer tetragalloyl, procyanidin hexamer galloyl, procyanidin heptamer, and the galloyl derivative (mean MW = 2052). The mean molecular weight was determined on the basis of the relative abundance of each flavanol in the fractions.

**Fluorescence Quenching and Nephelometry Measurements.** The quenching effect between α-amylase and procyanidin fractions with different molecular weights was assayed in the presence of increasing concentrations of different polysaccharides (arabic gum, β-cyclodextrin, and pectins with different methoxylation degrees—27, 58−63, and 72%) present in foods or added during food processing (*27*−*31*). A Perkin-Elmer LS 45 fluorometer was used for fluorescence quenching and nephelometry measurements. For the fluorescence quenching assays the excitation wavelength was set to 282 nm and the emission spectrum was recorded from 300 to 450 nm. Both slits were 10 nm. For nephelometry analysis, the fluorometer was used as a 90° light scattering photometer; for that, both excitation and emission wavelengths selected were the same (400 nm). At this wavelength, protein, tannins, and polysaccharides do not absorb the incident light (*32*).

Considering the possibility of fluorescence resonance energy transfer (FRET) between the protein and the procyanidin fractions, the absorption spectra of both were analyzed (data not shown): procyanidin fractions have an absorption maximum at 270 nm and decrease significantly until near 310 nm. The protein's emission spectrum starts at 320 nm, and at this $\lambda$ the polyphenols practically do not absorb. On the other hand, procyanidin fractions have their emission maximum at 330 nm, whereas the protein's absorption spectrum is between 200 and 290 nm. Thus, it does not seem possible that energy transference between these two molecules may occur.

The experiments were performed in 100 mM acetate buffer with 12% ethanol/water (v/v) as solvent (pH 5.0), and stock solutions of α-amylase (100 μM), procyanidin fractions (1 mM), and polysaccharides were prepared in this buffer. A pH of 5.0 was chosen because it is already known that α-amylase interactions with polyphenol are high at this pH, and it is close to mouth pH (7.0) (*33*).

Because the reactivity of procyanidins toward α-amylase depends on their MW, the concentration of each procyanidin fraction corresponds to the maximum aggregation for a fixed concentration of α-amylase (1 μM). Fraction I interacted weakly with α-amylase and was rejected in this study. The concentrations used (fraction II = 120 μM; fraction III = 50 μM; fraction IV = 17 μM) were obtained by a nephelometry curve, corresponding to the aggregation with increasing concentration of procyanidins (data not shown). The α-amylase concentration was chosen to be in the linear range of concentration with fluorescence.

In several microtubes, a volume of procyanidin fraction stock solution was mixed with different volumes (to make a final volume of 250 μL) of acetate buffer. After this, different volumes of the polysaccharide stock solution were added, and the tubes were mixed. The mixture was allowed to stand for 30 min, and the blank was measured (only for nephelometry) before the addition of 4 μL of the α-amylase stock solution. After the addition of α-amylase, the mixture was mixed and allowed to react for 1 h. After this, the microtube was mixed and the emission spectra and intensity of aggregates were measured in the fluorometer cell.

The effect of polysaccharide on the protein's fluorescence intensity was previously analyzed. For this, a mixture of α-amylase (1 μM) with the highest concentration of each polysaccharide (0.6 g $L^{-1}$ for arabic gum, 4.4 g $L^{-1}$ for cyclodextrin, and 80 mg $L^{-1}$ for all pectins) was prepared, and after 1 h, the protein's fluorescence intensity was measured.

Because the procyanidin fractions possess intrinsic fluorescence at $\lambda_{ex}$ (282 nm), their spectrum was measured and subtracted in all fluorescence

experiments. The effect of polysaccharides in the procyanidin fractiond emission spectra was also evaluated; it was observed that polysaccharides did not affect the procyanidin fractions spectra.

All assays were performed in triplicate.

**Dynamic Light Scattering (DLS) Measurements.** The size of aggregates present in solution was determined by DLS. The size of procyanidin fractions/PPA aggregates and the effect of the carbohydrates were measured using the Zetasizer Nano ZS Malvern instrument. This technique measures the time-dependent fluctuations in the intensity of scattered light that occur because particles undergo Brownian motion. The analysis of these intensity fluctuations enables the determination of the diffusion coefficients of particles, which are converted into a size distribution. The sample solution was illuminated by a 633 nm laser, and the intensity of light scattered at an angle of 173° was measured by an avalanche photodiode. This analysis provides information concerning particle size (obtained by the parameter intensity) and polydispersity.

These experiments were performed in triplicate.

**Statistical Analysis.** All assays were performed in $n = 3$ repetitions. The mean values and standard deviations were evaluated using analysis of variance (ANOVA); all statistical data were processed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com).

## RESULTS AND DISCUSSION

Fluorescence quenching of α-amylase/tannin complexes in the presence of increasing concentrations of polysaccharides was followed simultaneously with the measure of α-amylase/tannin aggregates by nephelometry. As the nephelometry measurements of aggregation are affected by several factors, mainly by the size of aggregates, this aspect was also evaluated by DLS technique.

**Effect of Arabic Gum on PPA/Procyanidin Fraction Complexation. Figure 1** shows the fluorescence emission spectrum (at $\lambda_{ex}$ = 282 nm) obtained for PPA and procyanidin fraction IV (mean MW = 2052) solution in the absence and increasing concentrations of arabic gum (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 g $L^{-1}$). Similar spectra were obtained with procyanidin fractions II and III (data not shown). As the concentration of arabic gum rises, the intensity of fluorescence increases.

The effect of different polysaccharides on the protein's fluorescence intensity was assayed to determine if the observed variations in fluorescence intensity were only due to modifications

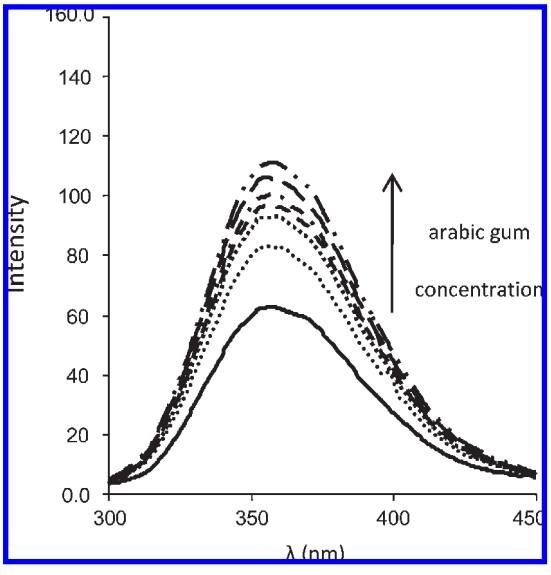


**Figure 1.** Fluorescence emission spectrum (at $\lambda_{ex}$ = 282 nm) of PPA (1 μM) and procyanidin fraction IV (17 μM; mean MW = 2052) solution, in the absence (full line) and presence of increasing concentrations of arabic gum (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 g $L^{-1}$). The experiments were performed in 100 mM acetate buffer (pH 5.0) with 12% ethanol/water (v/v).

of procyanidin interaction with α-amylase (**Figure 2a**). In general, the different polysaccharides studied did not affect significantly the α-amylase intrinsic fluorescence. On the other hand, procyanidins have been shown to have much lower fluorescence intensity than the one observed for proteins at the $\lambda_{ex}$ (**Figure 2b**). Nevertheless, procyanidin fraction spectra were measured and subtracted from the emission spectra of α-amylase. Therefore, the results obtained in the fluorescence quenching assays are only due to modifications in the interaction between protein and the quencher (procyanidin). This means that the quenching effect of procyanidins on PPA fluorescence decreases in the presence of arabic gum. However, the increase in fluorescence does not reach the protein's intrinsic fluorescence itself (~200 of intensity, in the same experimental conditions without procyanidins and arabic gum) even for higher concentrations of polysaccharide (data not shown). The increasing percentages of intrinsic fluorescence by the presence of arabic gum for all tested procyanidin fractions are shown in **Figure 3** (upper data). All procyanidin fractions followed the same behavior.

Overall, arabic gum was shown to be more efficient in reducing the quenching effect of PPA for the more highly polymerized fraction (IV).

For the same solution, the formation of aggregates (macromolecules) between α-amylase and procyanidins, which are supposed to be in part insoluble, was also followed by nephelometry (**Figure 3**, lower data). For all of the procyanidin fractions tested, a decrease in aggregation with the increase in arabic gum concentration was observed. This decreasing intensity of aggregation seems to be greater for the highly polymerized fractions. These results are in agreement with previous works (*19*, *34*).

It is interesting to observe that the aggregation decreases concomitantly with the increase of fluorescence. The competition between arabic gum and α-amylase for procyanidins leads to the decrease of the quenching effect and, consequently, to less aggregation.

**Effect of β-Cyclodextrin on PPA/Procyanidin Fraction Complexation.** The influence of β-cyclodextrin concentration in procyanidin/PPA intrinsic fluorescence is similar to that observed with arabic gum (**Figure 1**). The variation of intrinsic fluorescence and aggregation is shown in **Figure 4**.

As observed for arabic gum, fluorescence in the presence of β-cyclodextrin increases concomitantly with the decrease in percentage of aggregation. With regard to the quenching effect, the lower MW fraction (II) is the most affected, whereas the higher MW fraction (IV) is the less affected, oppositely to arabic gum, where fraction IV was the most affected fraction.

For arabic gum and β-cyclodextrin assays, the interaction between procyanidins (quenchers) with PPA is restrained by the presence of these polysaccharides, reducing the quenching effect and consequently reducing the aggregation, as confirmed by nephelometry.

Altogether, these results provide strong evidence for a molecular association in solution between polysaccharides and procyanidins, competing for protein aggregation and inhibiting the formation of aggregates.

Comparison of these two polysaccharides shows that arabic gum is by far the most effective in restraining the interaction between PPA and procyanidins, because the amount required was about 10-fold smaller than that of β-cyclodextrin. This is probably due to the polysaccharide structures, because β-cyclodextrin has a cone-shaped structure conferring conformational restrictions for the interaction with procyanidins. Effectively, β-cyclodextrin is a cyclic oligosaccharide, composed of seven 1→4-linked α-D-glucopyranoside units. The interior of the structure is less hydrophilic than the surface, being able to host

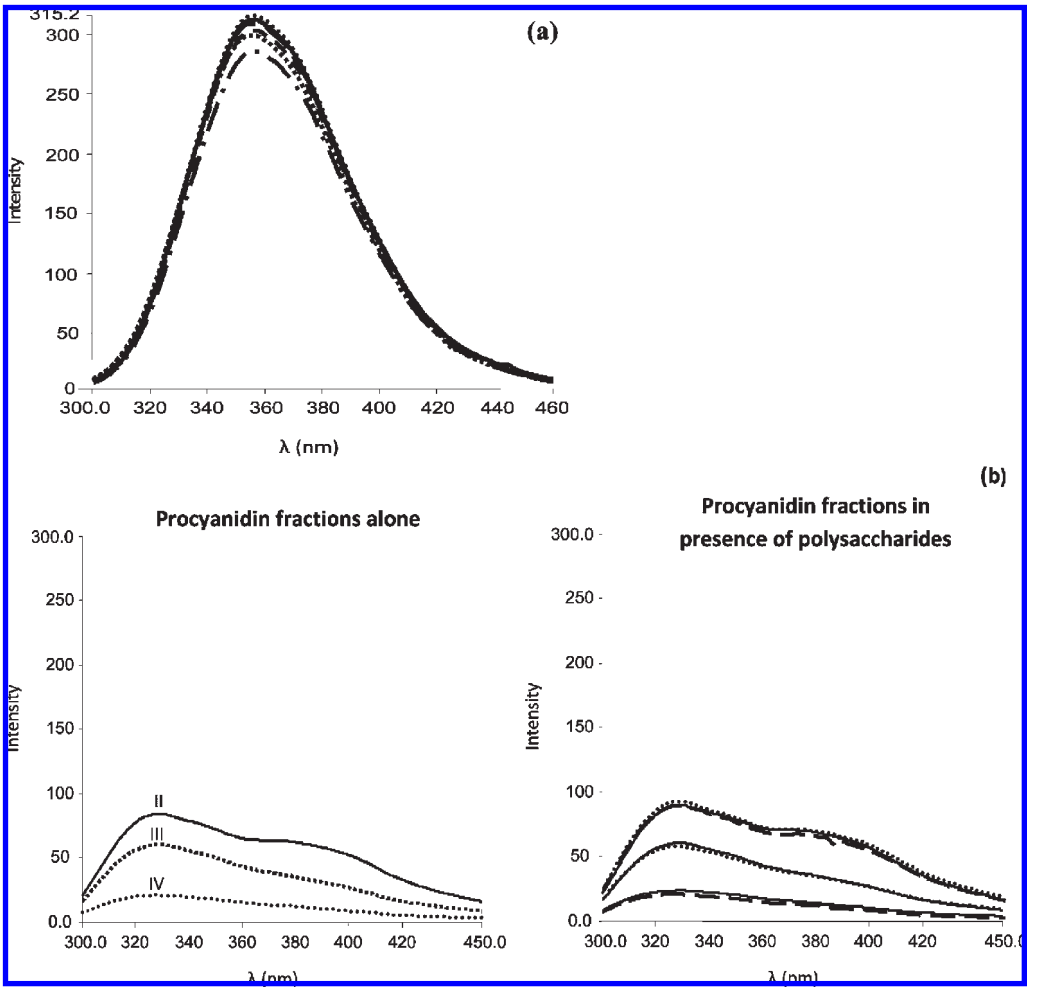


**Figure 2.** (**a**) Fluorescence emission spectrum (at $\lambda_{ex}$ = 282 nm) of PPA (1 $\mu$M) (solid spectra) and in the presence of the highest polysaccharide concentration [arabic gum 0.6 g $L^{-1}$, ···; $\beta$-cyclodextrin 4.4 g $L^{-1}$, - - -; and pectins 80 mg $L^{-1}$ (27% MD, — · —; 58−63% MD, — — —; and 72% MD, ——]. (**b**) Several fluorescence emission spectra of procyanidin fractions alone and in the presence of the highest polysaccharide concentration (arabic gum 0.6 g $L^{-1}$, ···; $\beta$-cyclodextrin 4.4 g $L^{-1}$, —; and pectin 58−63% MD 80 mg $L^{-1}$, — — —). The experiments were performed in 100 mM acetate buffer (pH 5.0) with 12% ethanol/water (v/v).

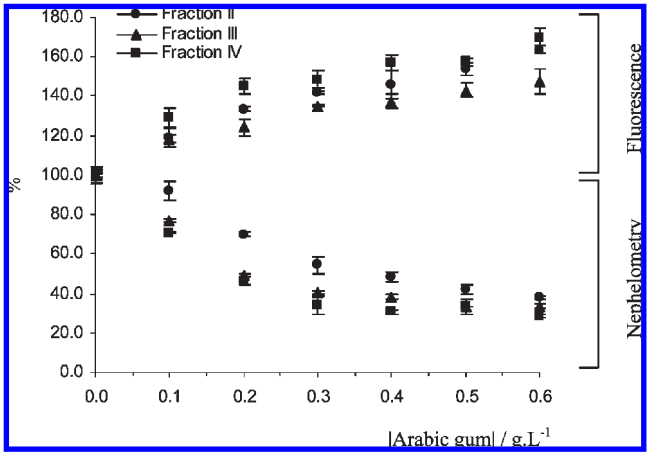


**Figure 3.** Influence of increasing concentrations of arabic gum in (upper data) variation of the fluorescence of PPA (1 $\mu$M) and procyanidin fractions solution and (lower data) percentage of procyanidin/PPA (1 $\mu$M) insoluble aggregates measured by nephelometry. Mean MWs of fractions II, III, and IV are 950, 1512, and 2052, respectively. All experiments were performed in 100 mM acetate buffer (pH 5.0) with 12% ethanol/water (v/v).

hydrophobic molecules in its interior (*30*, *31*). However, some studies (*35*, *36*) have shown that only small molecules, such as (+)-catechin, can be included inside the cyclodextrin cavity, which is not the case with procyanidin oligomers. On the other hand, arabic gum is a heteropolysaccharide composed by a polysaccharide and hydroxyproline-rich protein moieties able to ensure hydrophobic interactions with procyanidins (*27*, *28*). This polysaccharide moiety has also a slight acidic character, resulting from the presence of only 20% of glucuronic acids. Its more acidic character compared to $\beta$-cyclodextrin may help to establish electrostatic and cooperative hydrogen bonds that strengthen the interaction with procyanidins. This may explain the higher efficiency of arabic gum in restraining the aggregation between procyanidin fractions and PPA.

These kinds of electrostatic and ionic interactions with proteins and tannins have previously been shown with pectins from wines (*32*, *37*).

The restraining effect of both polysaccharides in the aggregation between procyanidin fractions and α-amylase is lower for fraction II than for the other two fractions. The effect of polysaccharides in aggregation seems to be affected differently with the increase in procyanidin MW. This effect was greater for fractions IV and III for arabic gum and $\beta$-cyclodextrin, respectively.

**Effect of Pectin with Different MDs on PPA/Procyanidin Fraction Complexation.** The variation in intrinsic fluorescence of α-amylase/procyanidin with different concentrations of

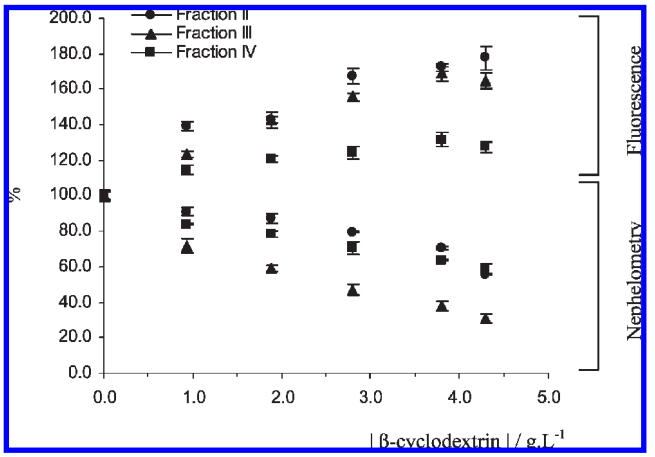


**Figure 4.** Influence of increasing concentrations of $\beta$-cyclodextrin in (upper data) variation of the fluorescence of PPA (1 $\mu$M) and procyanidin fractions solution and (lower data) percentage of procyanidin/PPA (1 $\mu$M) insoluble aggregates measured by nephelometry. Mean MWs of procyanidin fractions II, III, and IV are 950, 1512, and 2052, respectively. All experiments were performed in 100 mM acetate buffer (pH 5.0) with 12% ethanol/water (v/v).

pectins is shown in **Figure 5** (upper data). Oppositely to arabic gum and $\beta$-cyclodextrin, the fluorescence remains more or less constant with the increase of pectin concentration. In these cases, the quenching effect of α-amylase by procyanidins is not affected by the presence of pectins: the molecular environment in the vicinity of the chromophore (tryptophan residues) seems to remain unchanged.

The nephelometry analysis showed a systematic decrease in aggregation with the increase in pectin concentration, similar to that with arabic gum and $\beta$-cyclodextrin. Once again, this effect was lower for the procyanidin fraction with lower MW (II).

It is interesting to observe that although the effect of procyanidins in α-amylase fluorescence is not affected by the presence of pectins, their aggregation ability is importantly affected. Altogether, these results demonstrate that there is probably a molecular association between polysaccharides, procyanidin fraction, and protein in solution, resulting in a ternary complex and thereby forming more soluble aggregates in aqueous medium. Thus, the fluorescence quenching of PPA by procyanidin fractions is not reduced, but the formation of less soluble aggregates is still inhibited.

Pectins were the most effective polysaccharides that inhibited procyanidins/PPA aggregation, because the amount required was about a 10-fold lower concentration than that of arabic gum. This is probably due to their more acidic character and higher flexibility, when compared to the other polysaccharides tested. In fact, pectins are a very complex mixture of different polysaccharide molecules with a large range of molecular weights that vary with origin and extraction conditions. In general, they are composed by partially methylated poly-α-(1→4)-D-galacturonic acid residues (*38*). Pectins with high MD have reduced polarity, whereas pectins with low MD have a few hydrophobic groups.

However, no correlation between the pectins' MD and the restraining effect on procyanidin fractions/PPA aggregation was observed. Apparently, differences in the ratio of hydrophobic/hydrophilic groups between pectins did not seem to be enough to induce important different behaviors. Overall, among all the studied polysaccharides, pectins have a higher ionic character capable of establishing hydrophilic interactions.

**Size Measurement of Aggregates.** The measurement of the size of aggregates present in solution resulting from the interaction between the three procyanidin fractions (II, III, and IV) and PPA in the absence and presence of carbohydrates was performed by DLS. **Table 1** shows the average size ($Z$) of aggregates and also the polydispersity of solutions. In general, the polydispersity of solutions is below 0.5, which means that the $Z$ average size and polydispersity will give values that can be used for comparative purposes.

Despite the decrease of procyanidin fraction concentration relative to the molecular weight, the aggregates present in solution have approximately the same size, particularly for procyanidin

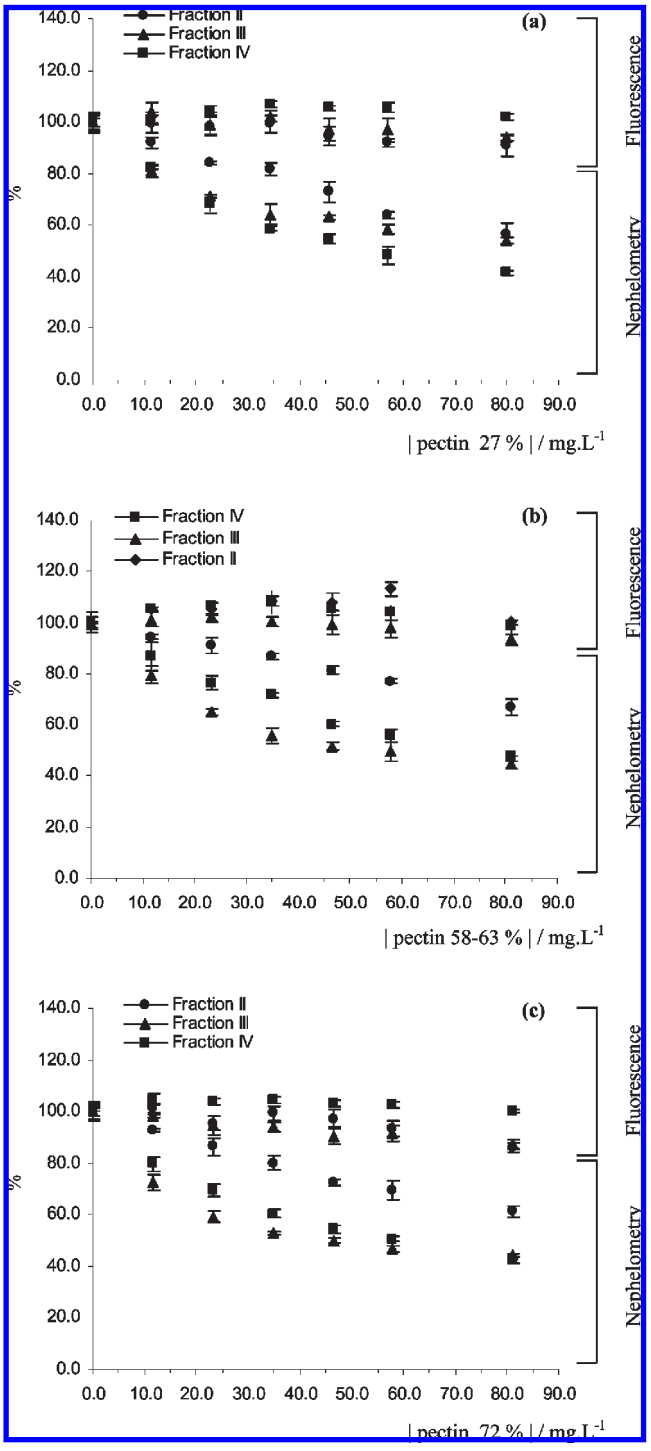


**Figure 5.** Percentage of procyanidin/PPA (1 $\mu$M) insoluble aggregates in the presence of increasing concentrations of different pectins measured by nephelometry. Mean MWs of procyanidin fractions II (**a**), III (**b**), and IV (**c**) are 950, 1512, and 2052, respectively. All experiments were performed in 100 mM acetate buffer (pH 5.0) with 12% ethanol/water (v/v).

**Table 1.** Average Size of Aggregates ($Z$) and Polydispersity Present in Procyanidin Fractions (II, 120 $\mu$M; III, 50 $\mu$M; IV, 17 $\mu$M) and PPA (1 $\mu$M) Solutions [100 mM Acetate Buffer with 12% Ethanol/Water (v/v), pH 5.0] in the Absence and Presence of Carbohydrates, Measured by DLS

| | fraction II | | fraction III | | fraction IV | |
|---|---|---|---|---|---|---|
| carbohydrate | $Z$ (nm) | polydispersity | $Z$ (nm) | polydispersity | $Z$ (nm) | polydispersity |
| none | 646 ± 38 | 0.221 | 485 ± 49 | 0.121 | 465 ± 28 | 0.346 |
| $\beta$-cyclodextrin (4.5 g $L^{-1}$) | 646 ± 45 | 0.833 | 445 ± 26 | 0.279 | 416 ± 25 | 0.021 |
| arabic gum (0.6 g $L^{-1}$) | 123 ± 7 | 0.525 | 156 ± 2 | 0.246 | 107 ± 6 | 0.500 |
| pectin (80 mg $L^{-1}$) | | | | | | |
| MD 27% | 249 ± 15 | 0.495 | 177 ± 11 | 0.248 | 173 ± 10 | 0.477 |
| MD 58−63% | 289 ± 17 | 0.464 | 196 ± 12 | 0.287 | 164 ± 10 | 0.425 |
| MD 72% | 300 ± 18 | 0.707 | 178 ± 11 | 0.307 | 219 ± 13 | 0.477 |

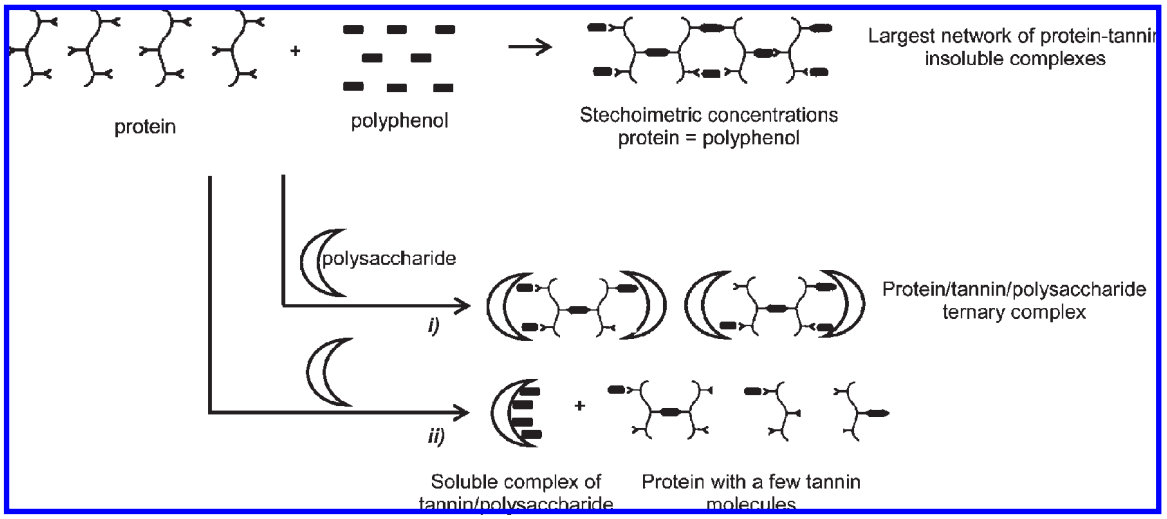


**Figure 6.** Possible mechanisms (i and ii) involved in the inhibition of the aggregation of tannins and proteins by polysaccharides. P, protein; T, tannin; C, polysaccharide/carbohydrate.

fractions III and IV (magnitude of 500 nm). This could be explained by the fact that the concentrations of procyanidin fractions used correspond to their maximum aggregation with PPA (stoichiometric concentrations), for which the increase in tannin concentration does not change aggregation (see Materials and Methods).

In general, the size of aggregates decreases in the presence of carbohydrates. Exceptionally, the one obtained for fraction II in the presence of $\beta$-cyclodextrin did not change. In that case, a large width of the particle size distribution was also observed (high polydispersity), indicating a very heterogeneous population of aggregates.

In the presence of pectin and especially of arabic gum there is a significant decrease in the size of aggregates, much higher than for $\beta$-cyclodextrin. These carbohydrates have induced a reorganization of the aggregates leading to the formation of smaller structures that reduce the scattered light, which is in agreement with the observed decrease of nephelometric measurements.

Therefore, as previously mentioned, the disrupting effect of polysaccharides toward protein/tannin aggregation has been proposed to result from two mechanisms (**Figure 6**): (i) the ability of polysaccharides to form a ternary complex protein/polyphenol/polysaccharide, thereby enhancing its solubility in aqueous medium, resulting in less insoluble aggregates; and (ii) the molecular association in solution between polysaccharides and polyphenols competing with protein aggregation.

From the results obtained herein, it can be proposed that the mechanisms i and ii, by which polysaccharides restrain the protein/polyphenol aggregation, are governed by the polysaccharide structure. The results obtained for arabic gum and $\beta$-cyclodextrin strongly suggest that the main mechanism by which these two compounds inhibit protein/polyphenol aggregation is mechanism ii (**Figure 6**). Arabic gum and $\beta$-cyclodextrin interact with procyanidin fractions, with more or less strength depending on procyanidin MW and also on the polysaccharide structure, and inhibit procyanidin interaction with PPA. Effectively, the quenching effect on α-amylase by procyanidins is decreased by the action of these polysaccharides promoting an increase in the observed intrinsic fluorescence intensity of α-amylase. Simultaneously, the aggregation decreases concomitantly with the increase of polysaccharide concentration.

The interaction between polysaccharides and polyphenols may result from cooperative hydrogen bonding, between the hydroxyl groups of polysaccharide and phenolics, and from hydrophobic interactions. The higher effect of arabic gum, compared to $\beta$-cyclodextrin, is probably due to its more acidic character, resulting from the presence of glucuronic acids able to establish more hydrogen bonds with procyanidins.

In the case of pectins, the results obtained provide substantial evidence for demonstrating that the main mechanism by which these compounds inhibit protein/polyphenol aggregation is mechanism i. Pectins seem to interact with procyanidin fractions and with PPA to form a ternary complex protein/polyphenol/polysaccharide, thereby enhancing its solubility in aqueous medium, resulting in smaller and more soluble aggregates. Effectively, opposite to arabic gum and $\beta$-cyclodextrin, the environment of the tryptophan residues of α-amylase is not affected by the presence of pectins, so the intrinsic fluorescence is not changed while solubilization occurs. This could mean that procyanidins still interact with α-amylase even in the presence of pectins.

Using DLS, it was observed that carbohydrates induce a decrease in aggregates size. The decrease in nephelometry intensity seems to be due to the formation of smaller aggregates, which results from the disruption and reorganization of the aggregates.

Overall, the combination of different analytical techniques—fluorescence quenching, nephelometry, and DLS—allowed important insights into the two mechanisms by which different polysaccharides are thought to inhibit protein/tannin aggregation. This development may help further investigations regarding the influence of carbohydrates in food taste.

## ABBREVIATIONS USED

PRP, proline-rich protein; PPA, porcine pancreas α-amylase; MD, methoxylation degree; DLS, dynamic light scattering; FRET, fluorescence resonance energy transfer.

## ACKNOWLEDGMENT

We thank Prof. Graham Jones from the University of Adelaide (Australia) for scientific advice and grammatical corrections.

## LITERATURE CITED

(1) Khanbabaee, K.; van Ree, T. Tannins: classification and definition. *Nat. Prod. Rep*. **2001**, *18*, 641–649.
(2) Haslam, E. *Plant Polyphenols—Vegetable Tannins Revisited—Chemistry and Pharmacology of Natural Products*; Cambridge University Press: Cambridge, U.K., 1989.
(3) Siebert, K. J.; Troukhanova, N. V.; Lynn, P. Y. Nature of polyphenol−protein interactions. *J. Agric. Food Chem*. **1996**, *44*, 80–85.
(4) Gonçalves, R.; Soares, S.; Mateus, N.; de Freitas, V. Inhibition of trypsin by condensed tannins and wine. *J. Agric. Food Chem*. **2007**, *55*, 7596–7601.
(5) Bennick, A. Interaction of plant polyphenols with salivary proteins. *Crit. Rev. Oral Biol. Med*. **2002**, *13*, 184–196.
(6) Griffiths, D. W. The inhibition of digestive enzymes by polyphenolic compounds. *Adv. Exp. Med. Biol*. **1986**, *199*, 509–516.
(7) Bate-Smith, E. C. Astringency in foods. *Food* **1954**, *23*, 124–127.
(8) Haslam, E. The association of proteins with polyphenols. *J. Chem. Soc., Chem Commun*. **1981**, 309–311.
(9) Lesschaeve, I.; Noble, A. C. Polyphenols: factors influencing their sensory properties and their effects on food and beverage preferences. *Am. J. Clin. Nutr*. **2005**, *81* (Suppl.), 330S–335S.
(10) Lu, Y.; Bennick, A. Interaction of tannin with human salivary proline-rich proteins. *Arch. Oral Biol*. **1998**, *43*, 717–728.
(11) Yan, Q.; Bennick, A. Identification of histatins as tannin-binding proteins in human saliva. *Biochem. J*. **1995**, *311*, 341–347.
(12) de Freitas, V.; Mateus, N. Nephelometric study of salivary protein−tannin aggregates. *J. Sci. Food Agric*. **2001**, *82*, 113–119.
(13) Mateus, N.; Pinto, R.; Ruão, P.; de Freitas, V. Influence of the addition of grape seed procyanidins to Port wines in the resulting reactivity with human salivary proteins. *Food Chem*. **2004**, *84*, 195–200.
(14) Elodis, P.; Móra, S.; Krysteva, M. Investigation of the active center of porcine-pancreatic amylase. *Eur. J. Biochem*. **1972**, *24*, 577–582.
(15) Buisson, G.; Duee, E.; Haser, R.; Payan, F. Three dimensional structure of porcine pancreatic α-amylase at 2.9 Å resolution. Role of calcium in structure and activity. *EMBO J*. **1987**, *6*, 3909–3916.
(16) Pasero, L.; Mazzei-Pierron, Y.; Abadie, B.; Chicheportiche, Y.; Marchis-Mouren, G. Complete amino acid sequence and location of the 5 disulphide bridges in porcine pancreatic α-amylase. *Biochim. Biophys. Acta* **1986**, *869*, 147–157.
(17) Ozawa, T.; Lilley, T. H.; Haslam, E. Polyphenol interaction: astringency and the loss of astringency in ripening fruit. *Phytochemistry* **1987**, *26*, 2937–2942.
(18) McManus, J. P.; Davis, K. G.; Beart, J. E.; Gaffney, S. H. Polyphenol interactions. Part 1. Introduction; some observations on the reversible complexation of polyphenols with proteins and polysaccharides. *J. Chem. Soc., Perkin Trans. 2* **1985**, 1429–1438.
(19) de Freitas, V.; Carvalho, E.; Mateus, N. Study of carbohydrate influence on protein−tannin aggregation by nephelometry. *Food Chem*. **2003**, *81*, 503–509.
(20) Carvalho, E.; Póvoas, M. J.; Mateus, N.; de Freitas, V. Application of flow nephelometry to the analysis of the influence of carbohydrates on protein−tannin interactions. *J. Sci. Food Agric*. **2006**, *86*, 891–896.
(21) Papadopoulou, A.; Green, R. J.; Frazier, R. A. Interaction of flavonoids with bovine serum albumin: a fluorescence quenching study. *J. Agric. Food Chem*. **2005**, *53*, 158–163.
(22) 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.
(23) 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.
(24) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*, 2nd ed.; Kluwer Academic/Plenum Publishers: New York, 1999.
(25) de Freitas, V. A. P.; Glories, Y.; Laguerre, M. Incidence of molecular structure in oxidation of grape seed procyanidins. *J. Agric. Food Chem*. **1998**, *46*, 376–382.
(26) Gonzalez-Manzano, S.; Mateus, N.; de Freitas, V. A. P. Santos-Buelga, C. Influence of the degree of polymerisation in the ability of catechins to act as anthocyanin copigments. *Eur. Food Res. Technol*. **2008**, *227*, 83–92.
(27) Kjøniksen, A.; Hiorth, M.; Roots, J.; Nyström, B. Shear-induced association and gelation of aqueous solutions of pectin. *J. Phys. Chem. B* **2003**, *107*, 6324–6328.
(28) Goodruma, L. J.; Patelb, A.; Leykamc, J. F.; Kieliszewski, M. J. Gum arabic glycoprotein contains glycomodules of both extensin and arabinogalactan-glycoproteins. *Phytochemistry* **2000**, *54*, 99–106.
(29) Sanchez, C.; Renard, D.; Robert, P.; Schmitt, C. Structure and rheological properties of acacia gum dispersions. *Food Hydrocolloids* **2002**, *16*, 257–267.
(30) Biwer, A.; Antranikian, G.; Heinzle, E. Enzymatic production of cyclodextrins. *Appl. Microbiol. Biotechnol*. **2002**, *59*, 609–617.
(31) Saenger, W.; Jacob, J.; Gessler, K.; Steiner, T.; Hoffmann, D.; Sanbe, H.; Koizumi, K.; Smith, S. M.; Takaha, T. Structures of the common cyclodextrins and their larger analogues—beyond the doughnut. *Chem. Rev*. **1998**, *98*, 1787–1802.
(32) Carvalho, E.; Mateus, N.; Plet, B.; Pianet, I.; Dufourc, E.; de Freitas, V. Influence of wine pectic polysaccharides on the interactions between condensed tannins and salivary proteins. *J. Agric. Food Chem*. **2006**, *54*, 8936–8944.
(33) Young, G.; Resca, H. G.; Sullivan, M. T. The yeasts of the normal mouth and their relation to salivary acidity. *J. Dent. Res*. **1951**, *30*, 426–430.
(34) Mateus, N.; Carvalho, E.; Luís, C.; de Freitas, V. Influence of the tannin structure on the disruption effect of carbohydrates on protein−tannin aggregates. *Anal. Chim. Acta* **2004**, *513*, 135–140.
(35) Jullian, C.; Miranda, S.; Zapata-Torres, G.; Mendizábal, F.; Olea-Azar, C. Studies of inclusion complexes of natural and modified cyclodextrin with (+)-catechin by NMR and molecular modeling. *Bioorg. Med. Chem*. **2007**, *15*, 3217–3224.
(36) Kriz, Z.; Koča, J.; Imberty, A.; Charlot, A.; Auzély-Velty, R. Investigation of the complexation of (+)-catechin by β-cyclodextrin by a combination of NMR, microcalorimetry and molecular modeling techniques. *Org. Biomol. Chem*. **2003**, *1*, 2590–2595.
(37) Vernhet, A.; Pellerin, P.; Prieur, C.; Osmianski, J.; Moutounet, M. Charge properties of some grape and wine polysaccharide and polyphenolic fractions. *Am. J. Enol. Vitic*. **1996**, *47*, 25–30.
(38) Pérez, S.; Rodríguez-Carvajal, M. A.; Doco, T. A complex plant cell wall polysaccharide: rhamnogalacturonan II. A structure in quest of a function. *Biochimie* **2003**, *85*, 109–121.

**Received for Review October 15, 2008. Revised manuscript received March 27, 2009. Accepted April 02, 2009. This research was supported by research project grant (PTDC/AGR-ALI/67579/2006) funding from FCT (Fundação para a Ciência e Tecnologia) from Portugal.**