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Influence of Carbohydrates on the Interaction of Procyanidin B3 with Trypsin

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Supporting Information

ABSTRACT: The biological properties of procyanidins, in particular their inhibition of digestive enzymes, have received much attention in the past few years. Dietary carbohydrates are an environmental factor that is known to affect the interaction of procyanidins with proteins. This work aimed at understanding the effect of ionic food carbohydrates (polygalacturonic acid, arabic gum, pectin, and xanthan gum) on the interaction between procyanidins and trypsin. Physical−chemical techniques such as saturation transfer difference-NMR (STD-NMR) spectroscopy, fluorescence quenching, and nephelometry were used to evaluate the interaction process. Using STD-NMR, it was possible to identify the binding of procyanidin B3 to trypsin. The tested carbohydrates prevented the association of procyanidin B3 and trypsin by a competition mechanism in which the ionic character of carbohydrates and their ability to encapsulate procyanidins seem crucial leading to a reduction in STD signal and light scattering and to a recovery of the proteins intrinsic fluorescence. On the basis of these results, it was possible to grade the carbohydrates in their aggregation inhibition ability: XG > PA > AG ≫ PC. These effects may be relevant since the coingestion of procyanidins and ionic carbohydrates are frequent and furthermore since these might negatively affect the antinutritional properties ascribed to procyanidins in the past.

KEYWORDS: trypsin, procyanidin, carbohydrates, STD-NMR, fluorescence quenching

INTRODUCTION

Procyanidins (PC) are secondary metabolites of plants that are abundant in fruits and vegetable-based beverages like juices, beer, and wine.[1] They are polymeric ramified structures composed of flavan-3-ols with elementary units linked by C−C and occasionally C−O−C bonds.[2] Dimeric procyanidins are composed of catechin and epicatechin linked through C4−C8 and C4−C6 bonds.

Procyanidins have the ability to interact with proteins, in particular digestive enzymes that make them worthy of attention by diverse areas such as medicine, toxicology, chemistry, food science, and agriculture. This interaction has been studied using nephelometry,[3,4] fluorescence quenching,[5,6] dynamic light scattering,[7] isothermal titration calorimetry,[8,9] mass spectrometry,[10] small-angle X-ray scattering,[11] and nuclear magnetic resonance,[10,12−15] among others. It has been found that environmental factors such as pH, ionic strength, nature of the solvent, and presence of carbohydrates largely affect the interaction process.[3,16] The effect of carbohydrates is particularly relevant since in vegetable food matrixes, procyanidins and carbohydrates are often found together. Two distinct mechanisms have been proposed to explain how procyanidin/protein interaction is affected by carbohydrates (Figure 1):[14]

(i) They could form a ternary complex with the already associated procyanidin and protein and simply increase their solubility in aqueous medium.

(ii) Carbohydrates may directly compete with the protein for the binding of procyanidins thereby shifting the balance toward more free protein. In some cases, this may involve an encapsulation of polyphenol in a gel-like matrix.

Some ionic carbohydrates such as pectin, arabic gum, and polygalacturonic acid are able to prevent the association between procyanidins and proline-rich proteins from saliva by what appears to be a competition mechanism.[3,4,14] So far, their effect on the association of procyanidins with globular proteins with enzymatic activity has only been scarcely studied.[2]

One of the mechanisms for the biological activities of polyphenols, including procyanidins, is their inhibition of digestive enzymes. Trypsin (PPT, EC 3.4.21.4) is a digestive proteolytic enzyme that presents several isoforms and exists in mammals. It is included in the group of the serine proteases whose activity is deregulated in diseases such as platelet aggregation disorders, rheumatoid arthritis, pancreatitis, cystic fibrosis, pulmonary emphysema, and asthma.[17] Apart from this, a reduction in the activity of trypsin, that is responsible for the hydrolysis of proteins into peptides and amino acids, may be harmful since it can contribute to a reduced absorption of nutrients. PPT also plays a central role in the digestive process because it is not only responsible for its autoactivation (by proteolysis of a zymogen) but also for the activation of other classes of digestive enzymes.[18]

PPT inhibition by polyphenols of different sources has been studied in a previous work, and a relationship between galloylation and inhibition was observed for the monomeric flavanols in tea[19−21] and between the degree of polymerization and inhibition for procyanidins.[22] In the case of procyanidins, it was found that oligomers of molecular weights above 1400 (five or more catechin units) are very good inhibitors of the enzyme and that below this weight there is no significant difference in inhibitory efficacy.[22,23]

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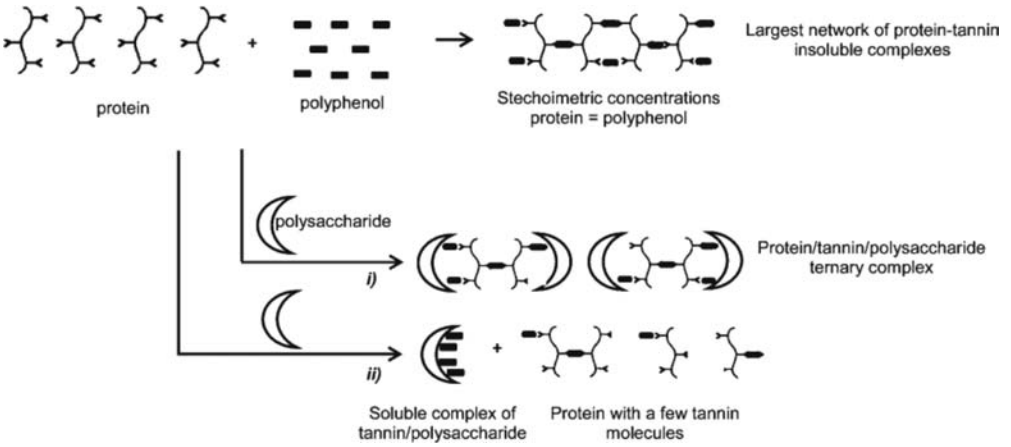

Figure 1. Possible mechanisms (i and ii) involved in the inhibition of the aggregation of tannins and proteins by carbohydrates/polysaccharide. P, protein; T, tannin; and C, polysaccharide/carbohydrate.[6]

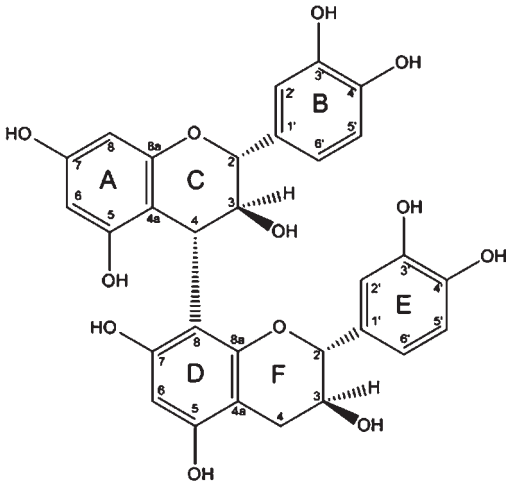

Figure 2. Structure of procyanidin dimer B3 and its respective ^{1}H chemical shifts (δ).

The effect of dietary carbohydrates on the biological activities of procyanidins has been largely overlooked. Therefore, the objective of this work was to determine the effect of some food carbohydrates (polygalacturonic acid, arabic gum, pectin, and xanthan gum) in the association of procyanidin B3 with trypsin using saturation transfer difference-NMR (STD-NMR) spectroscopy. One of the most widely studied procyanidins is dimer B3 (catechin-(4$\beta \rightarrow$ 8)-catechin; Figure 2) due to its high abundance in the human diet and relevant antioxidant activity.[24] STD-NMR was used as the main source of information on the interaction of procyanidin B3 with trypsin. These studies were complemented by other techniques such as fluorescence quenching and nephelometry that are widely used to study the interaction between tannins and proteins.[4,5]

MATERIALS AND METHODS

Reagents. Trypsin IX-S from a porcine pancreas, (+)-catechin hydrate, polygalacturonic acid (PA), arabic gum (AG), pectin (PC) from citrus fruits, and xanthan gum (XG) were purchased from Sigma Aldrich (St. Louis, USA). (+)-Taxifolin was purchased from Extrasynthèse (Genay, France).

Synthesis and Purification of Procyanidin B3. Procyanidin B3 was synthesized according to the procedure described in the literature with slight modifications.[25,26] Taxifolin (200 mg) and catechin (575 mg) were dissolved in ethanol under argon atmosphere. Sodium borohydride (in ethanol) was slowly added to the mixture. pH was lowered to 4.5 with CH_3COOH/H_2O 50% (v/v), and the mixture was allowed to stand under argon atmosphere for 30 min. The reaction mixture was extracted with ethyl acetate, evaporated, and passed through C18 gel. Following extensive washing with water, procyanidins were recovered with methanol, which was evaporated. The obtained fraction was passed through a TSK Toyopearl HW-40(s) gel column (300 mm $\times$ 10 mm i.d., with 0.8 mL min^{-1} methanol as the eluent) coupled to a UV−vis detector (Gilson 115). Several fractions were recovered and analyzed by ESI-MS (Finnigan DECA XP PLUS) yielding procyanidins with varying degrees of polymerization. The fraction containing procyanidin B3 ($[M - H]^-$ at m/z = 577) was isolated and freeze-dried. The total yield after purification was 22% procyanidin B3.

Analysis and Purification of Carbohydrates. All carbohydrates were 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 μm filters) and dried. Pectin was analyzed by colorimetric methods and gas chromatography to determine sugar composition and esterification degrees.[27,28] It was found to be composed by 85% galacturonic acid; 10% galactose; and 5% of other sugars. The degree of methylation and acetylation was determined to be 14% and 1%, respectively.

NMR Studies. Protein samples containing 160 μM PPT were prepared in D_2O and divided into several 5 mm NMR tubes in order to maintain the protein concentration constant throughout the experiments. For the same reason, both procyanidin B3 and carbohydrate samples were lyophilized and added as a powder, allowing for the same NMR tube to be used for all the experiments with each carbohydrate.

Procyanidin B3 was added at a concentration of 4.8 mM allowing for a molar ratio of 1:30 (PPT/procyanidin B3). Polygalacturonic acid (PA) was added in the 0.2−1.0 g/L range, arabic gum (AG) was added in the 0.2−1.6 g/L range, pectin (PC) was added in the 2−10 g/L range, and xanthan gum (XG) was added in the 0.001−0.09 g/L range.

Several assays at different concentrations of carbohydrates were performed in order to establish the concentration range where their effect is more pronounced and effective. The maximum concentrations used were limited by carbohydrate solubility in the NMR solvent. Only the results for these concentration ranges were shown.

NMR experiments were performed on a Bruker AVANCE III 400 spectrometer equipped with a 5 mm gradient inverse broadband probe with a deuterium lock. ^{1}H and STD spectra were recorded with a shaped

pulse to suppress the water resonance[29] using the following parameters: spectral width, 20 ppm; nutation angle (90°), 7.0 μs; and 90° shaped pulse duration, 2 ms.

Selective saturation of protein off-resonance at 25 ppm and on-resonance at 0.5 ppm was conducted using a pseudo-two-demensional (2D) sequence for STD with a shaped pulse train alternating between the on- and off-resonances.[30,31] The spectra were collected in an interleaved fashion to reduce temporal fluctuations.[32] As controls, the experiment was performed on solutions of procyanidin B3 or protein alone to confirm that the on- and off-resonance irradiation frequencies did not affect the procyanidin and that the protein was not saturated by the off-resonance irradiation. STD-NMR spectra were acquired at 298 K using EBurp-shaped pulses for selective saturation (50 ms), with a 2.5 μs delay between pulses, which corresponds to a total saturation time of approximately 2.5 s. The number of scans (24), receptor gain value (2050), and relaxation delay (3.5 s) were kept constant for quantitative purposes. Software (TopSpin 2.1; Bruker, Newark, DE) was used to subtract the unprocessed on- and off-resonance spectra (both FIDs in the pseudo-2D experiment), baseline-correct the resulting difference spectrum, and integrate the areas of procyanidin B3 peaks. In the titration experiments, the relevant peaks (H2′, H5′, and H6′ of rings B and E) were integrated, and the integrals were summed. To ensure the specificity of STD eliciting resonances, another STD-NMR experiment was recorded for the same sample (trypsin and procyanidin B3) in which a capillary containing a 1 mM glucose solution was inserted. Since T1 relaxation may be affected by solution viscosity, procyanidin B3 T1 relaxation times were determined in the presence of the largest carbohydrate concentration tested. It was found that small changes were induced in T1, but the relaxation times remained high. It is therefore valid to assume that there are no changes in the STD effect due to T1 relaxation changes.

Fluorescence Quenching. Changes in the quenching of PPT fluorescence by procyanidin B3 in the presence of the different carbohydrates were assayed using a Perkin-Elmer LS 45 fluorimeter. Tryptophan was used as an intrinsic fluorophore (λ_{ex} = 290 nm; λ_{em} = 340 nm). The λ_{ex} was set to 290 nm, and the emission spectrum was recorded from 300 to 500 nm. A pH of 7.0 (50 mM phosphate buffer) was chosen because it is the one that occurs in the duodenum after the neutralization of gastric fluids.[33] In several microtubes, procyanidin B3 (90 μM) was added to PPT (3 μM) to achieve the same molar ratio that was used in the NMR experiments. A smaller concentration of protein was required since this fluoroscopic method is more sensitive to PPT than STD-NMR. The ratios between protein and carbohydrate concentration was maintained at the same values as those of the STD-NMR experiment. After successive addition of increasing volumes of stock solution of each carbohydrate, the microtube was shaken, and the emission spectra were measured in the fluorimeter cell. Since procyanidins are known to possess intrinsic fluorescence λ_{ex} (282 nm), its spectrum in the presence of each carbohydrate was measured and subtracted in all fluorescence experiments. For each experiment, a relative fluorescence value (%) was calculated as the ratio between the fluorescence of the measured sample and that of the unquenched protein (100%).

Bearing in mind the risk of fluorescence resonance energy transfer (FRET) between protein and procyanidin, the absorption spectra of both were examined (data not shown): procyanidin B3 has an absorption maximum at 270 nm that decreases considerably until near 310 nm. The protein's emission spectrum starts at 320 nm, and at this λ, procyanidin B3 virtually does not absorb. However, B3 has an emission maximum at 330 nm, while the protein's absorption spectrum is between 200 and 290 nm. Therefore, it does not seem likely that energy transference between these two molecules may occur.

Nephelometry Measurements. Nephelometry experiments were conducted as the fluorescence quenching assays except for the detection method. During nephelometry, a Perkin-Elmer LS 45 fluorimeter was used as a 90° light scattering photometer;[34] both excitation and emission wavelengths selected were the same (400 nm). At this wavelength, protein, tannins, and polysaccharides do not absorb the incident light.[34] A relative aggregation value (%) was calculated for each experiment as the ratio between the scatter intensity of the measured sample and that of the most turbid sample (100%) containing PPT and B3 without carbohydrates.

Statistical Analysis. All assays were performed in $n \geq 3$ repetitions. Values are expressed as the arithmetic means ± SEM. Statistical significance of the difference between groups was evaluated by one-way analysis variance (ANOVA) followed by Tukey's test. Differences were considered significant when $P < 0.05$. Data were processed using GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, California, USA).

RESULTS

The influence of several carbohydrates on the interaction between trypsin and procyanidin B3 was investigated by STD-NMR, fluorescence, and nephelometry. Experiments were conducted for four different ionic carbohydrates: polygalacturonic acid (PA), arabic gum (AG), pectin (PC), and xanthan gum (XG). Ionic carbohydrates were chosen since, according to previous work, they are highly effective in reducing protein/tannin aggregation.[3,6]

A molar ratio of procyanidin B3/PPT of 30:1 was chosen to conduct all the experiments with carbohydrates because it was shown previously that at this stoichiometry, B3 is capable of causing a reduction in both trypsin fluorescence and activity as well as to elicit resonances in STD experiments (submitted work).

A reference STD-NMR experiment was conducted on a solution containing 160 μM PPT and 4.8 mM B3 (Figure 3). The saturation of the protein at 0.5 ppm is visible in the on-resonance spectrum (Figure 3A), which is different from the off-resonance spectrum (Figure 3B). The latter is similar to a regular ^{1}H spectrum of the mixture (not shown). In the STD sequence used, the difference spectrum is obtained (Figure 3C) by subtraction of the on-resonance spectrum from the off-resonance. It is visible that the saturation that was induced in trypsin at 0.5 ppm was transferred essentially to the aromatic protons of procyanidin B3 that appear well-defined in the 5.5−7.0 region of the STD difference spectrum. To verify if the STD conditions were well established, an external capillary with glucose was placed into the NMR tube in contact with the procyanidin B3 and trypsin solution. As expected, it was observed that the signals corresponding to glucose are not present in the same STD spectrum where the resonances of procyanidin B3 were visible (Figure S1, Supporting Information). This indicates the unequivocal specificity of the binding of procyanidin B3 to the studied protein and the functionality of the STD pulse sequence to study specific interactions.

To the same solution containing 160 μM PPT and 4.8 mM procyanidin B3, increasing concentrations of polygalacturonic acid (0.2−1 g/L), arabic gum (0.2−1.6 g/L), pectin (2−10 g/L), and xanthan gum (0.001−0.09 g/L) were added.

Preliminary assays at different concentrations of carbohydrates were performed in order to establish the concentration range where their effect is more pronounced and effective. Maximum concentrations of carbohydrates were also limited by their solubility. Only the results for these concentration ranges were shown.

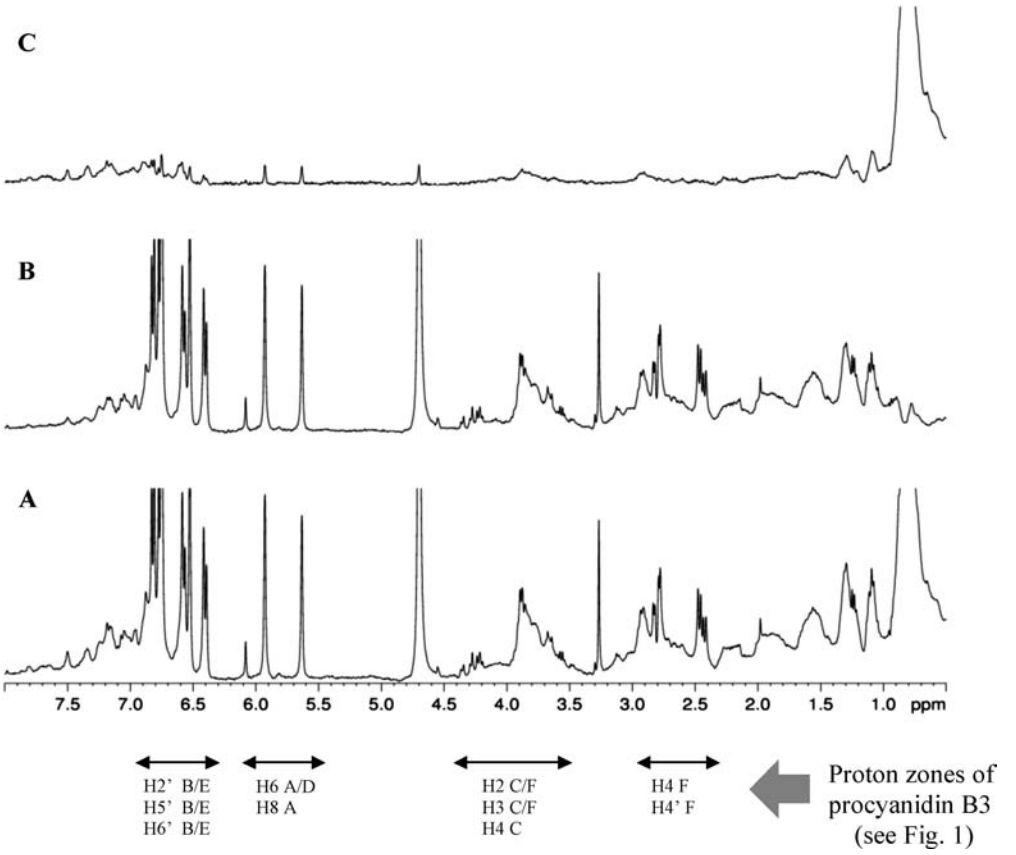

Figure 3. STD-NMR experiment of the interaction between trypsin (160 μM) and B3 (4.8 mM) recorded in D_2O: A, on-resonance STD-NMR ^1H; B, off-resonance STD-NMR ^1H; C, difference spectrum (B subtracted from A).

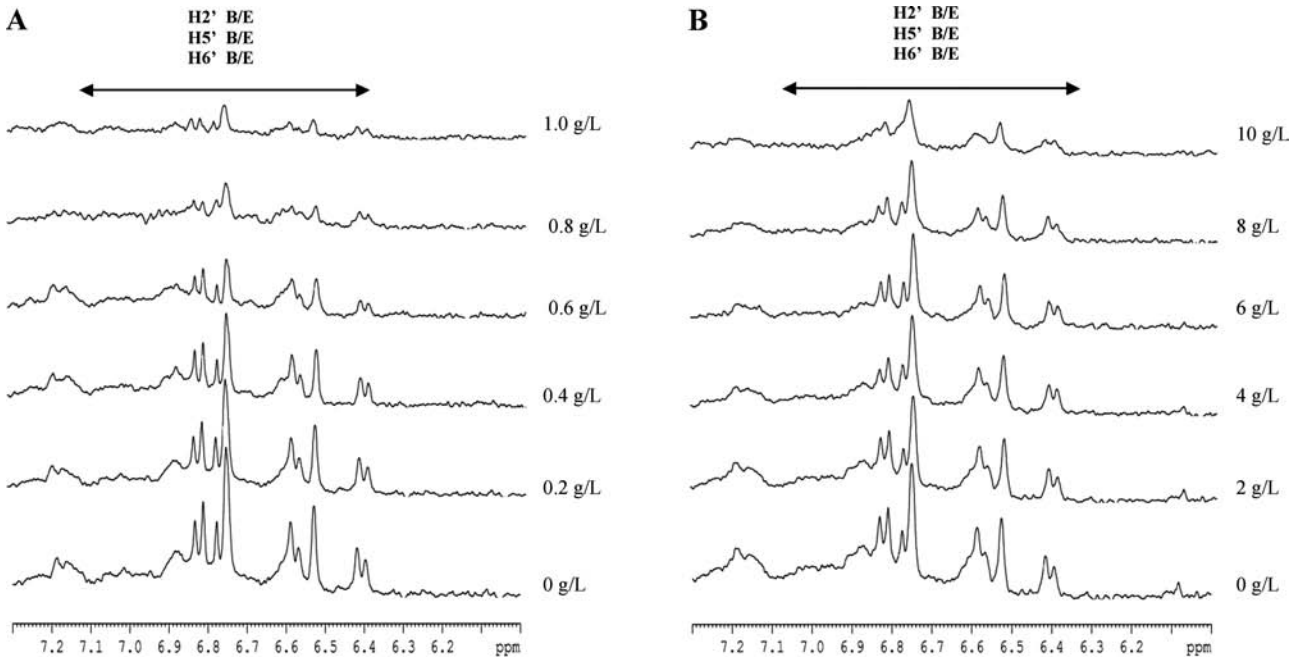

Figure 4. Representative spectra from an STD experiment on a solution of PPT (160 μM) and procyanidin B3 (4.8 mM) with an increasing concentration of polygalacturonic acid, 0.2−1 g/L (A), and pectin, 2−10 g/L (B), where the decrease in intermolecular transferred NOE is visible.

All carbohydrates caused a relevant modification of the STD signals of the procyanidin/protein solution (Figure 4). The signals in the 5.7 to 6.2 ppm range (corresponding to H6 A, H8 A, and H6 D) were excluded in the titration curves because

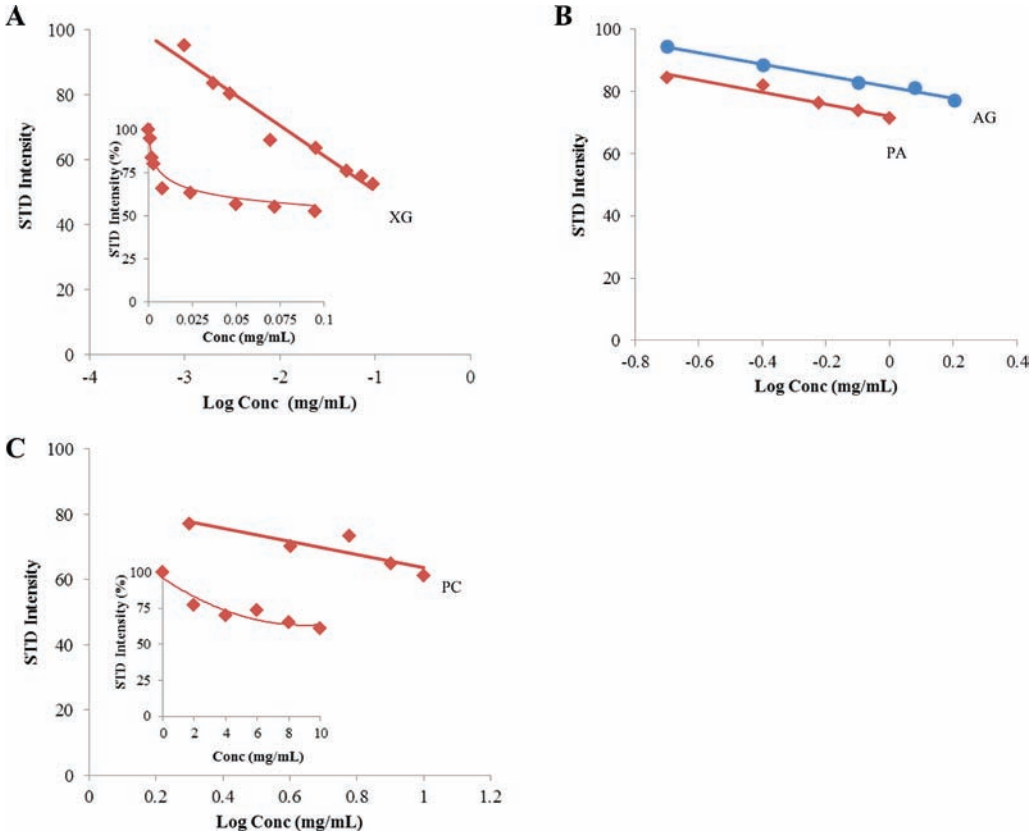

Figure 5. Variation (%) in STD signal intensity (graphical insert) and respective logarithmic plot of procyanidin B3 (4.8 mM) in the presence of PPT (160 μM) with increasing concentrations of different carbohydrates: A, xanthan gum (red diamond); B, polygalacturonic acid (red diamond) and arabic gum (blue circle); C, pectin (red diamond).

they exchange with D from the solvent D_2O over time. In general, carbohydrates induced a reduction in the STD signals between 6.4 and 7.0. This could mean that carbohydrates induced a dissociation of the PPT/B3 complexes by competition with B3.

The intensities of the sum of protons (H2′, H5′, and H6′ of rings B and E) in the STD spectra were represented as a function of the carbohydrate concentration (Figure 5). In the graphical insert of Figure 5A, it is possible to observe a decrease in proton intensity with the increase of the concentration of xanthan gum. There is an initially fast decrease of the signal intensity for lower concentrations of xanthan gum after which further addition of carbohydrates slowly affects only the decrease of proton intensity. The application of a semilogarithmic treatment to the data yields a linear plot also indicating the typical saturation behavior (Figure 5A). Similar plots were obtained for all other carbohydrates in the concentration range tested (Figure 5B,C).

The slope of the semilogarithmic plots in Figure 5 may be used as a measure of the sensitivity of the STD effect to carbohydrates and therefore was chosen to compare the effect of different carbohydrates. On the basis of this slope, it is possible to rate the ability of each carbohydrate to interfere with the association of procyanidin/protein in the order XG > PA > AG ≫ PC.

To supplement the information obtained in the STD-NMR experiments at different concentrations, fluorescence quenching and nephelometry studies were conducted. Quenching of protein fluorescence by procyanidins has been used to study the effects of procyanidins on enzyme structure in particular regarding the environment near the chromophore molecule. The most common chromophore in proteins is tryptophan. As previously reported,[23] the addition of procyanidins to trypsin causes a fluorescence quenching effect reducing the intensity of the proteins' emission maximum. This reduction is attributed to an association between procyanidins and trypsin that, assuming the same behavior that was seen in STD-NMR, may be disrupted by carbohydrates.

Because of the higher sensitivity of the fluorescence technique, the concentration of trypsin (3 μM), procyanidins, and carbohydrates were reduced accordingly to keep the molar ratios studied by NMR. Blank assays were conducted with carbohydrates in the absence of procyanidin B3, and no variation of protein fluorescence was induced by carbohydrates.

The plots of relative fluorescence intensity vs log (concentration of carbohydrate) for B3/trypsin solutions are shown in Figure 6. In these plots, 100% corresponds to the maximum fluorescence intensity of trypsin in the absence of B3. In the presence of procyanidin B3 and in the absence of carbohydrates, trypsin fluorescence decreases about 23% in these experimental conditions. In general, in the presence of carbohydrates, trypsin recovers some fluorescence intensity, which confirms that the carbohydrates have the capacity to reduce the quenching ability of B3 toward trypsin. This effect is more pronounced for XG, AG, and PA (Figure 6A,B). For pectin, the restitution of fluorescence to the enzyme is residual with only a very slight variation from the fully quenched state, even for much higher concentrations than the other carbohydrates (Figure 6C). A saturation behavior was observed for XG, PA, and AG in a manner similar to that of the

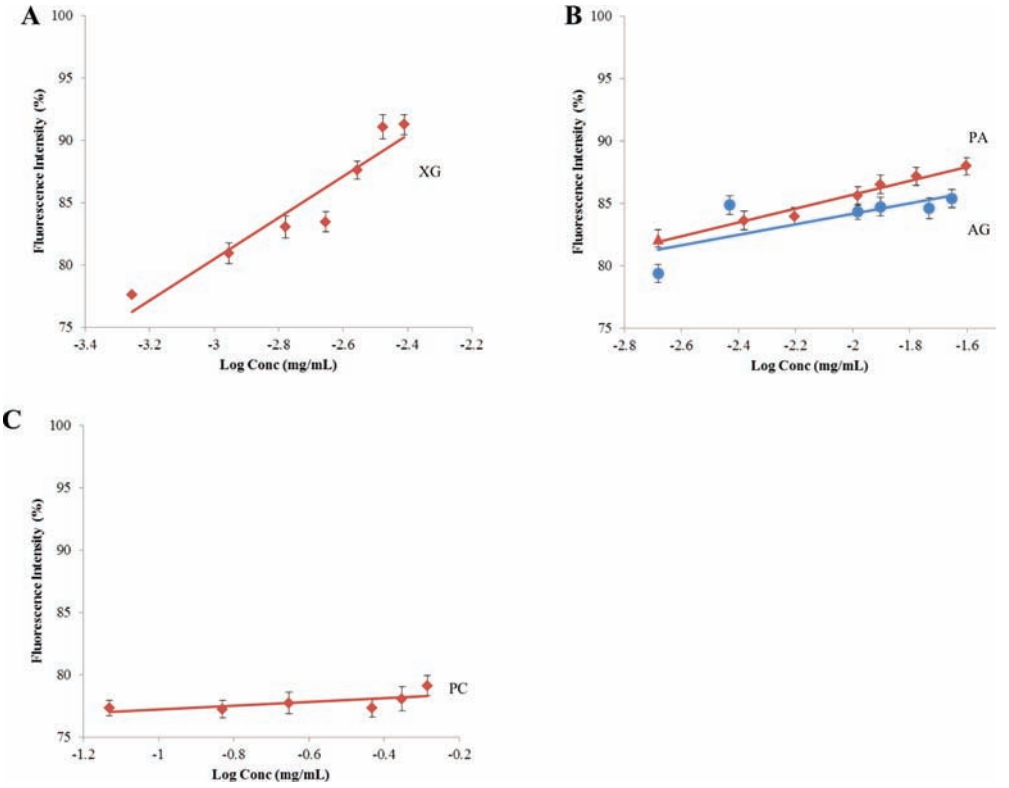

Figure 6. Variation (%) in fluorescence intensity at 346 nm of PPT (3 μM) and procyanidin B3 (90 μM) with increasing concentrations of different carbohydrates: A, xanthan gum (red diamond); B, polygalacturonic acid (red diamond) and arabic gum (blue circle); C, pectin (red diamond).

STD-NMR; the slopes of the semilogarithmic plot were also used to compare the effectiveness of carbohydrates in dissociating B3/trypsin aggregates. These results are in agreement with the ones obtained by STD-NMR. Indeed, the carbohydrates with the higher slopes in the semilog plots also induce a greater recovery in trypsin fluorescence. The value obtained with pectin clearly illustrates the lower effectiveness of this carbohydrate.

Both STD-NMR and fluorescence quenching evaluate the first stages of the interaction between procyanidin B3 and trypsin molecules. However, it is well-known that the interaction between these compounds also leads to an aggregation process during the following stages of the interaction between tannin and protein (supramolecular level). As such and bearing this in mind, the measurement of the aggregates in solution was estimated by nephelometry (Figure 7). A relative aggregation value (%) was calculated as the ratio between the scatter intensity of each sample and that of the most turbid sample (100%) containing only PPT and B3.

In general, it is possible to observe a decrease of aggregation in the presence of carbohydrates, which may correspond to either the solubilization or more probably the dissociation of the B3/trypsin aggregates. The curves obtained show an initial stage of aggregation reduction that tends to stabilize with the increase in carbohydrate concentration.

Strong dissociation/solubilization of aggregates (20% initial aggregation) was observed for XG (Figure 7A) and PA (Figure 7B). AG and PC (Figure 7C) seem to affect aggregates to a lesser extent with 45% and 85% of aggregation, respectively. Generally, these results are in agreement with those obtained by STD-NMR and fluorescence quenching. Nevertheless, some subtle differences (more evident stabilization behavior for all carbohydrates and an effect of PA closer to XG than in STD and fluorescence) in the ability of carbohydrates to disrupt PPT/B3 aggregates as measured by nephelometry and by the other techniques may result from the way that interactions occur at molecular (STD-NMR and fluorescence) and supra-molecular (nephelometry) levels.

DISCUSSION

The results obtained with the three techniques indicate that all carbohydrates are capable of reducing the interaction between trypsin and procyanidin B3 and their aggregation. Fluorescence and STD-NMR techniques clearly show that there is a dissociation of the trypsin/procyanidin aggregates upon the addition of carbohydrates. Taken together, these results indicate that a competition mechanism between carbohydrates and trypsin is occurring leading to a shift in the balance toward more free protein (increase in fluorescence) and less bound procyanidin to trypsin (decrease in STD signal) (Figure 8).

One of the main features that could be responsible for the ability of carbohydrates to compete with trypsin for the binding of procyanidins is their ability to encapsulate procyanidins. XG consists of a linear β-D-glucose backbone with charged trisaccharide side chains that adopt a gel-like network in solution by lateral association of ordered chain sequences with the ability to encapsulate phenolic compounds (Figure 8).[35] In fact, this ability was demonstrated in the past with procyanidins and appears to be favored when low molecular weight procyanidins are used, probably because the size of the pores formed is small.[36] Therefore, the highly charged XG is able to establish hydrogen bonds with polyphenols that help to stabilize the resulting complexes. Polygalacturonic acid, much like XG, has both a

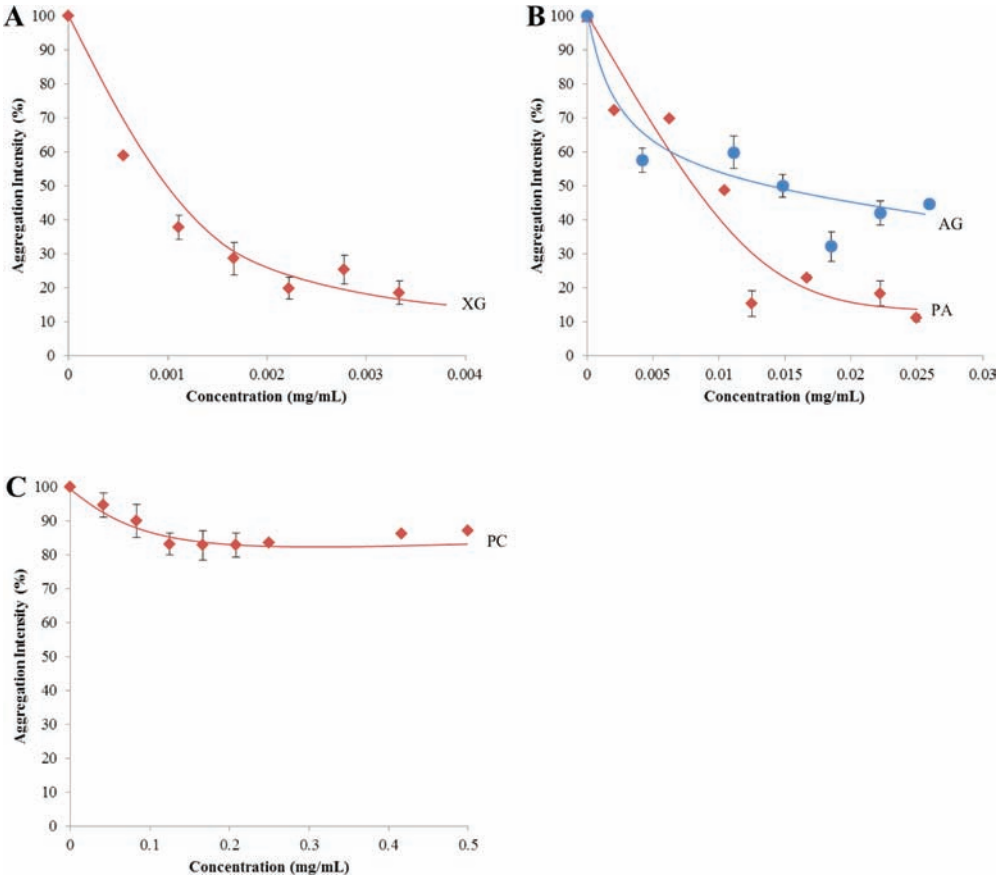

Figure 7. Variation (%) in light scattering (aggregation of PPT and procyanidin B3) at 400 nm of PPT (3 μM) and procyanidin B3 (90 μM) with increasing concentrations of different carbohydrates: A, xanthan gum (red diamond); B, polygalacturonic acid (red diamond) and arabic gum (blue circle); C, pectin (red diamond).

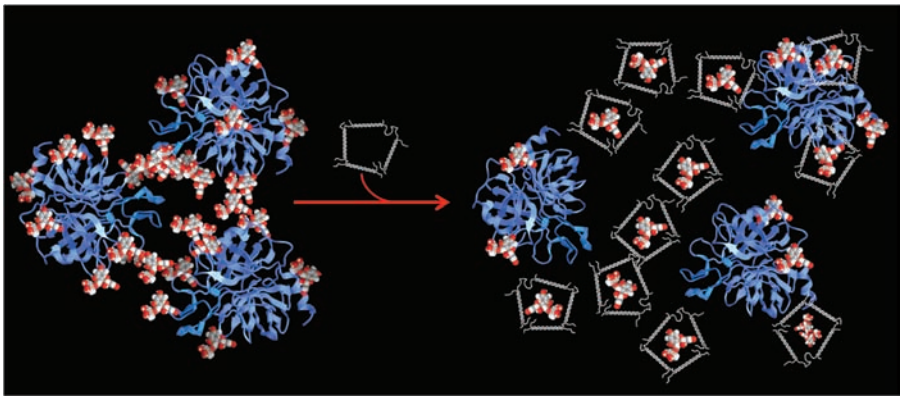

Figure 8. Competition effect of xanthan gum on PPT/B3 aggregates leading to their solubilization. XG, gray square; PPT, blue ribbon; B3 (CPK spheres). A similar mechanism is expected for the other carbohydrates.

charged character being composed solely of a linear (1-4)-poly-α-D-galacturonic acid chain and the ability to form hydrophobic molecular cavities that may encapsulate procyanidin B3 that may also be stabilized by hydrogen bonding.[37]

Both PC and AG have a much lower ionic character with the first being composed of an arabinogalactan-type polysaccharide with a few galacturonic acid residues and the latter being a methoxylated derivative of PA and therefore of a lower ionic character. In the buffer solution used, the coiled structure of AG may be dissociated by the presence of salts making this carbohydrate adopt an unstructured form.[38] PC has the same ability as PA to form hydrophobic cavities; however, these are smaller (due to the presence of the methyl groups) and much more hydrophobic. These features probably explain why these carbohydrates are less effective in preventing the aggregation of procyanidins and trypsin.

In conclusion, all tested carbohydrates are capable of disrupting aggregates formed between procyanidin B3 and trypsin by a competition mechanism in which the ionic character of carbohydrates and their ability to encapsulate procyanidins account for the quantitative differences observed among them. From a food industry and nutrition perspective, these effects may be relevant since food polyphenols are usually ingested together with food carbohydrates. These effects may contribute to the reduction of the antinutritional effects ascribed to procyanidins during digestion involving enzymes such as trypsin and others.

■ ASSOCIATED CONTENT

Supporting Information. Classic proton and corresponding STD-NMR spectra of a solution of PPT and procyanidin B3 with 5 mM glucose in an external capillary. This material is available free of charge via the Internet at http://pubs.acs.org.

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