Structural Features of Procyanidin Interactions with Salivary Proteins

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Procyanidin dimers and trimer C1 were synthesized, whereas (−)-epicatechin *O*-gallate and B2−3′′-*O*-gallate were isolated from grape seeds. Human saliva was separated into two fractions. One of these was mainly α-amylase and the other mainly proline-rich proteins (PRPs). The procyanidin compounds were combined with each of the saliva protein fractions and with bovine serum albumin. The protein−polyphenol interactions were observed using nephelometry. (+)-Catechin had a higher tannin specific activity (TSA) for PRPs than (−)-epicatechin (1.45 versus 0.65 nephelos turbidity units/mg of polyphenol). This indicated the effect of the stereochemistry of flavan-3-ols on their interaction with proteins. Procyanidin dimers linked through a C(4)−C(8) interflavanoid bond had consistently greater TSA than their counterparts with a C(4)−C(6) linkage. Esterification of a galloyl group to the C(3) hydroxyl function of (−)-epicatechin or to the epicatechin moiety of procyanidin dimer B2 increased TSA. This was not as strong an effect for the dimer, probably as a result of the expected "closed" structure of B2−3′′-*O*-gallate.

Keywords: *Astringency; nephelometry; grape seeds; procyanidin; salivary proteins; BSA*

INTRODUCTION

Proanthocyanidins (condensed tannins) are polyphenol secondary metabolites that are widespread in the plant kingdom and able to precipitate proteins. This feature is at the origin of their harsh astringent taste. Furthermore, it is of particular interest in foodstuffs and beverages, especially in wines, for which astringency is seen as a major organoleptic property. The astringency phenomenon is thought to be due to the interaction of salivary proteins with polyphenols present in wine. This event will result in insoluble aggregates that precipitate, obstructing the palate lubrication and causing an unpleasant sensation of roughness, dryness, and constriction (*1*−*3*). Among salivary proteins, proline-rich proteins (PRPs), which represent ∼70% of the whole human salivary protein content, can strongly bind tannins, suggesting a crucial role in the phenomenon of astringency (*4*−*7*). PRPs have a proline content of 28−40% and this, together with glutamine and glycine, constitutes 75−80% of all amino acid residues (*8*). Tannin/PRP associations are supposed to involve face-to-face stacking of aromatic groups onto proline residues, whereas the interaction with globular proteins probably involves only surface exposure aromatic residues (*9*, *10*). Polyphenol complexation with proteins has been largely studied in solution [NMR spectroscopy (*9*), microcalorimetry (*11*), enzyme inhibition (*12*)] and by protein precipitation (*13*−*17*). Some works concerning the influence of the procyanidin structure involved in this activity have also been reported (*18*−*20*). Besides the relative proportion of polyphenol to protein, the interactions between proteins and procyanidins are affected by the solution conditions (solvent composition, ionic strength, pH, and temperature) (*21*−*27*). Additionally, carbohydrates may affect the binding ability of the condensed tannins because they influence the final protein−tannin complex solubility (*28*). The binding ability of tannins depends also on their molecular size and on the number of sites in the molecule able to associate with proteins. Within polyphenol compounds, procyanidins constitute a class with great structural diversity which could demonstrate stereospecific interactions with proteins (*12*, *29*−*33*). This can be approached from a variety of structural considerations including the shape, projection of phenolic hydroxyls, addition of galloyl groups, and position of peripheral groups imposed by the stereochemistry of the pyranic ring. Beart and co-workers have shown that for the galloyl-D-glucose series the strength of the association with bovine serum albumin (BSA) is enhanced with the addition of every galloyl ester group up to five units (*11*). On the other hand, they have shown that vascalagin and castalagin, structural analogues to penta-*O*-galloyl-D-glucose, bind weakly to BSA as a result of their rigid structures. To maximize the possibilities for polyphenol−protein interactions, it is important for the polyphenol molecule to be conformationally mobile.

In the work presented here, nephelometry was used to assay the influence of different structural factors of procyanidins, such as catechin units [(+)-catechin and (−)-epicatechin], interflavanoid bond linkage (C4−C8 and C4−C6), and gallic acid esterification, on their ability to bind salivary proteins (α-amylase and PRPs) and BSA in a model solution.

MATERIALS AND METHODS

Procyanidin Dimers and Trimers. The procyanidin dimers B1−B8 and trimer C1 (Figure 4) were synthesized as previously described (*34*, *35*). Catechins, procyanidin dimers, and trimer C1 were identified by analytical HPLC, by comparison with authentic standards, as described elsewhere (*36*).

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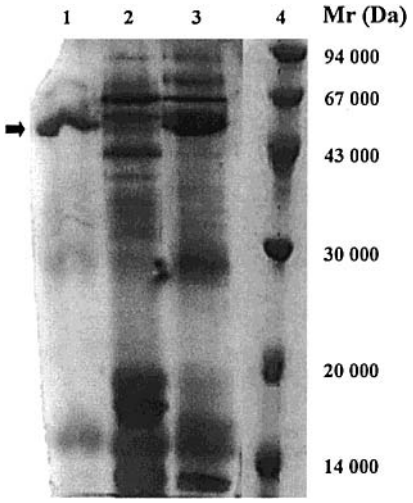

Figure 1. SDS-PAGE of crude human saliva and two fractions obtained after the protein purification process: lane 1, fraction rich in α-amylase; lane 2, fraction rich in PRPs; lane 3, saliva crude extract; lane 4, MW markers; the arrow indicates α-amylase.

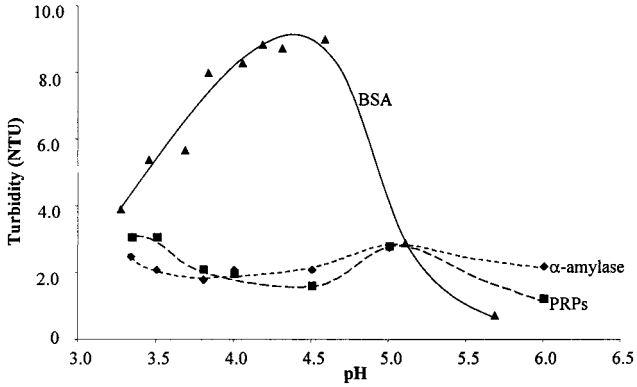

Figure 2. Influence of pH in the TSA of PRPs (48 μg), α-amylase (132 μg), and BSA (60 μg).

(−)-Epicatechin *O*-gallate and B2−3′-*O*-gallate were isolated by HPLC, and their structures were elucidated by NMR.

Bovine Serum Albumin. BSA was purchased from Sigma Chemical Co. (St Louis, MO).

Salivary Protein Purification. Saliva was collected from a volunteer who expectorated saliva into an ice-cooled tube. Saliva flow was induced by applying small quantities of lemon juice onto the volunteer's tongue. After collection, EDTA was added to a final concentration of 5 mM and the saliva samples were bulked and stored at −20 °C. Salivary proteins were fractionated using a combination of methods described in the literature with some minor modifications (*37*, *38*). All operations were performed at 2 °C. The whole saliva was dialyzed overnight against 50 mM KH_2PO_4 buffer, pH 6.8. Ammonium sulfate (biochemical grade) was added slowly to the dialyzed solution with stirring to achieve 45% saturation. The resultant protein suspension was stirred overnight and then centrifuged at 20000*g* for 30 min. The supernatant (from which the PRPs were purified) was dialyzed against several changes of 50 mM Tris-HCl buffer, pH 8.6, and then applied to a 17 × 1.6 cm column of DEAE-Sephadex A25 previously equilibrated with the same buffer. The column was eluted with starting buffer at 15 mL h^{-1}. The absorbance of the eluate was monitored at 230 nm. This first fraction yielded the unbound total basic fraction. After 250 mL had been collected, when all unbound protein had been eluted, 50 mM Tris-HCl, 0.5 M NaCl buffer, pH 8.6, was applied to the column to yield the acidic fraction. The unbound basic fraction was concentrated to 5 mL using a 50 mL Amicon stirred ultrafiltration cell with a 3 kDa cutoff membrane. The pellet from the ammonium sulfate precipitation described above was resuspended, dialyzed exhaustively against water, and lyophilized. The residue was dissolved in 50 mM KH_2PO_4 buffer, pH 8.0, and applied to a 17 × 1.6 cm column of DEAE-Sephadex A50 equilibrated in the same buffer. The column was eluted with starting buffer at 15 mL h^{-1}, and the absorbance was monitored at 230 nm. The eluate yielded the unbound α-amylase fraction. All of the final fractions from all of the columns were dialyzed exhaustively against water and lyophilized. Polyacrylamide gel electrophoresis was then performed over the resulting fractions of PRPs and α-amylase.

Table 1. TSA of Procyanidins (0.4 mg) with PRPs, α-Amylase, and BSA[a]

	TSA (NTU/mg of tannin)		
	BSA	α-amylase	PRPs
monomers			
(+)-catechin	nd[b]	0.42	1.45
(−)-epicatechin	nd	0.42	0.65
(−)-epicatechin *O*-gallate	nd	nd	2.25
dimers			
B2 *O*-gallate	nd	0.30	1.10
B2	nd	0.50	0.85
B3	nd	1.82	2.75
B4	nd	0.25	1.75
B6	nd	1.18	2.30
B7	nd	0.32	2.10
B8	nd	0.25	0.85
trimers			
C1 [(−)-epi-(−)-epi-(−)-epi]	nd	nd	1.05

[a] Data are means from quadruplicate assays (coefficient of variation < 0.10). *t* test experiments were performed for every mean that was not found to be significantly different ($p < 0.05$). [b] nd, not detected.

Protein Analysis by SDS-PAGE. All of the samples were mixed with an equal volume of electrophoresis sample buffer (0.125 M Tris-HCl, 4% SDS; 20% v/v glycerol, 0.02% bromophenol blue, pH 6.8) and heated at 100 °C for 5 min. These samples were thereafter analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis according to the procedure described in the literature (*39*) using 12.5% acrylamide separating gel. The stacking gel was 4% acrylamide, and the ratio between acrylamide and *N*,*N*′-methylenebis(acrylamide) was 29.2/0.8. The experiment was performed using a 15 mA/gel power supply for the stacking gel and 20 mA/gel for the separating gel. The gel was fixed with a mixture of ethanol, acetic acid, and deionized water (40:10:50) for 1 h. The gel was then washed with water for 5 min and soaked in a 10% gluteraldehyde solution for 30 min (to retain small proteins in the gel), followed by extensive deionized water washes. The proteins were stained with silver nitrate (*40*). Molecular weights were estimated by comparison with standard proteins (low molecular weight electrophoresis calibration kit, Amersham Pharmacia Biotech Inc., Piscataway, NJ).

Nephelometry. Nephelometry experiments were performed in a HACH 2100N laboratory turbidimeter. The optical apparatus is equipped with a tungsten filament lamp with three detectors: a 90° scattered-light detector, a forward-scatter light detector, and a transmitted light detector. Previous calibration was performed using a Gelex Secondary Turbidity Standard Kit (HACH), which consists of stable suspensions of a metal oxide in a gel. This analytical method requires ideal conditions where all particles are small and identical (*16*). A procyanidin solution of 2 mg L^{-1} in water, ethanol 12% v/v (pH 5.0), was prepared. Two milliliters of this solution was introduced in a test tube for each assay. Furthermore, the proteins were added and the mixture was shaken and stored at room temperature. The tannin specific activities (TSA) were expressed in nephelos turbidity units (NTU) per milligram of flavan-3-ol. All experiments were performed in quadruplicate. Concerning statistical analysis, *t* test experiments were performed for every mean in Table 1 using the SPSS computer package ($p < 0.05$).

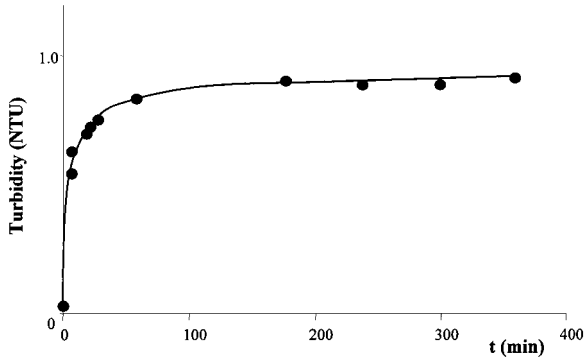

Figure 3. Time−rate profile of the appearance of insoluble protein−tannin aggregates in a solution (water/ethanol 12% v/v, pH 5.0) containing procyanidins (760 μg) and PRPs (48 μg).

RESULTS AND DISCUSSION

Procyanidin dimers and trimer C1 were synthesized, and previously purified human saliva was separated in two fractions: one with α-amylase and the other essentially PRPs (Figure 1). These proteins account almost entirely for the whole protein salivary content. The fraction containing the PRPs was found to be richer in low molecular weight (MW) proteins (between 15 and 20 kDa) than high molecular weight proteins. The proteins stained at the bottom of the gel correspond to histatins (histidine-rich proteins) (*41*). The analytical method used in this study—nephelometry—allows the measurement of scattered light in solution resulting from the gradual formation in time of a cloudy precipitate corresponding to the tannin−protein interaction. These interactions are importantly affected by the relative concentration of tannins and proteins and also by the solvent composition and pH (*27*, *42*).

First, the influence of pH in these interactions was studied. Procyanidin oligomers exhibited two maxima of highest capacity to bind and precipitate salivary proteins, especially PRPs at pH 3.5 and 5.0, and bind extensively BSA around pH 4.5 (Figure 2). From these results, the pH for following studies was set at 5.0 because it was the pH value nearest mucous secretion salivary glands (pH 5.6−7.9), at which proteins were shown to be stable and have a maximum ability to bind and precipitate procyanidin oligomers. The relative tannin specific activity (TSA) of procyanidins toward salivary proteins and commercial BSA was measured according to their ability to precipitate proteins in a model solution (water/ethanol 12% v/v, pH 5.0). Under these conditions, the controls showed that proteins were not precipitated by the solvent (ethanol 12%) in the absence of tannins and no precipitate was observed in the polyphenol solutions without any protein, using higher concentrations than those used in the experiments. Several assays were previously performed with the progressive addition of protein in order to obtain a maximum of insoluble tannin/protein aggregates at pH 5.0, from which no further precipitation occurred. The haze formation increased in time and started to stabilize after 40 min (Figure 3). The amounts found for each protein studied were 48, 60, and 132 μg for PRPs, BSA, and α-amylase, respectively. TSA values were set as the maximum turbidity point from which no further precipitation would occur with an excess of protein.

Influence of Molecular Structure. The binding capacity depends not only on the molecular size but also on the number and stereospecificity of separate sites on the molecule able to associate with proteins (*19*, *20*). This specificity of procyanidins can be approached from a variety of structural considerations including asymmetries of the side groups imposed by the chiral centers of pyranic rings, projections of phenolic hydroxyls, and the presence of galloyl groups. Therefore, procyanidin dimers and trimer C1 were synthesized, whereas (−)-epicatechin *O*-gallate and B2−3″-*O*-gallate were isolated from grape seeds (Figure 4). The influence of different structural factors of procyanidins, such as the catechin structure units [(+)-catechin and (−)-epicatechin], interflavanoid bond linkage [C(4)−C(6) and C(4)−C(8)], and gallic acid esterification on the relative TSA, defined here by its ability to bind and precipitate proteins, is shown in Table 1. Monomers, dimers, and trimer C1 were found to have higher affinities for PRPs than for α-amylase, whereas no insoluble aggregates were observed in the reaction with BSA. Although BSA was found to be unable to form insoluble aggregates with procyanidin monomers and dimers, it is more likely to react with procyanidin oligomers having a higher MW (data not shown). Therefore, BSA cannot be used to evaluate low MW tannin−protein interactions using this method. Previous nephelometric studies performed in our laboratory have already indicated that practically no globular protein precipitation occurred with low MW tannins such as monomers or monomer gallates. It has been shown that small polyphenols can bind to proteins but not cross-link them to generate haze (*43*). The expected higher affinity of PRPs can be explained by its randomly coiled structure with more active binding sites as compared to the globular conformations of BSA and α-amylase. It could also be that these globular proteins lack proline (at least on their surface), which has been shown to be required for polyphenol binding (*21*, *44*). The TSA toward salivary proteins appears to be related to the flavanol structure. (+)-Catechin had a higher TSA for PRPs compared to (−)-epicatechin (1.45 and 0.65, respectively). This result agrees with previous literature (*43*). This fact points out the importance of the pyranic ring stereochemistry of flavan-3-ols in their ability to interact with proteins.

Concerning procyanidin compounds, it should be noted that the TSA value found for dimer B3 [(+)-catechin-(4−8)−(+)-catechin] is practically 2-fold higher than the one obtained for (+)-catechin (2.75 and 1.45, respectively). This is presumably due to the doubling molecular size, resulting in more ortho phenolic groups and aryl rings able to bind and precipitate proteins without any major conformational restriction. This is not true for procyanidins with (−)-epicatechin as the main structural monomeric unit. Effectively, the TSA value per (−)-epicatechin unit increased only slightly with incremental degree of polymerization from monomer (0.65) up to dimer B2 (0,85) and trimer C1 (1.05). Although the number of active sites that may bind proteins increased proportionally with the number of (−)-epicatechin units, the resulting precipitation was not proportional, which may be explained, in part, by the conformational restraints imposed by the interflavanoid bond. The higher tannin affinity observed for C(4)−C(8) interflavanoid bond dimers B3 and B4 (2.75 and 1.75), compared to their respective C(4)−C(6) analogue dimers B6 and B8 (2.30 and 0.85), is possibly due to conformational changes imposed by the C(4)−C(6) interflavan bond, which may make difficult protein−tannin interac-

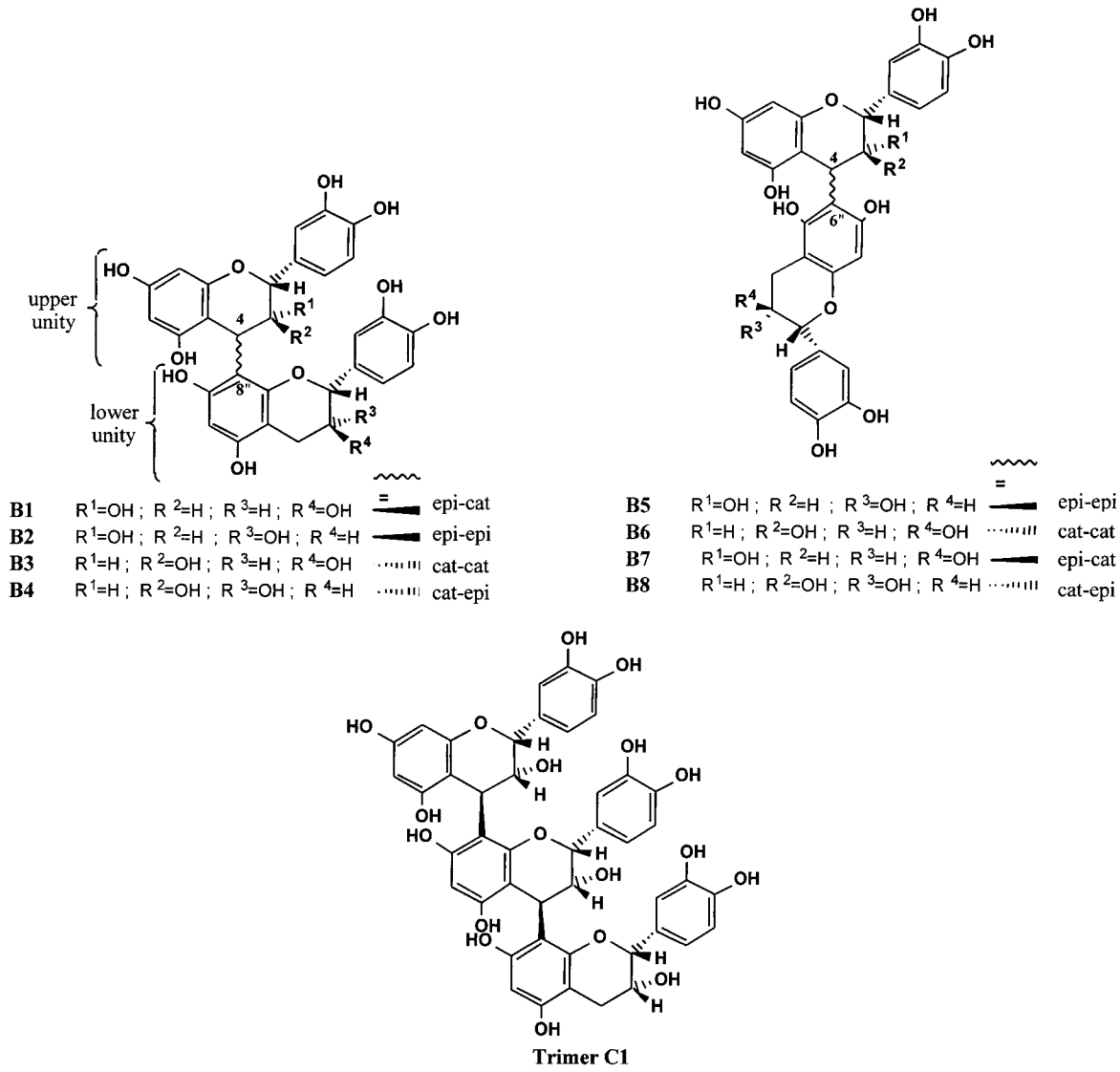

Figure 4. Molecular structures of procyanidin dimers (B1−B8) and trimer C1.

tions. These results reveal an important effect of the interflavanoid bond linkage and the influence of the structural monomeric units on procyanidin's ability to precipitate PRPs. Additionally, for procyanidin dimers containing the C(4)−C(6) interflavanoid bond, (+)-catechin as the lower monomeric unit increased their ability to bind PRPs; the TSA values of dimers B6 and B7 are nearly identical and higher than the one obtained for dimer B8. Differences in the TSA values of dimers appear thus to be related to their stereoisomeric differences, because their atoms will not be in the same direction on the periphery of the molecule. This feature will lead to different protein−tannin complex precipitation with different dimers.

Influence of Esterification with Gallic Acid. Esterification of (−)-epicatechin with gallic acid at the C(3) hydroxyl function increased importantly its capacity to form insoluble complexes with PRPs (from a TSA value of 0.65 to 2.25). This augmentation of ∼1.6 NTU/mg of flavan-3-ol may be explained by the fact that the (−)-epicatechin gallate molecule has a well-exposed galloyl function which enables binding to several sites on either the same peptide or in different peptide molecule through hydrophobic effects and hydrogen bonding. Despite the fact that the strength of the association of (−)-epicatechin with PRPs was shown to be enhanced with the addition of the galloyl ester group, this feature seemed to be less important for its analogue dimer (B2), for which the galloyl function increased the TSA only slightly from 0.85 (B2) to 1.10 (B2 gallate). This result might be explained by some loss of conformational freedom of the gallate structure of dimers compared with monomeric units such as (−)-epicatechin. In fact, previous molecular studies concerning the estimation of the flavan-3-ol conformation in solution using molecular mechanics and NMR techniques showed a possible $\pi-\pi$ stacking arrangement between the aromatic gallate and catechol rings of dimer gallates (Figure 5). Similar interactions are absent in their analogous dimeric procyanidins (*45*). This structural feature results in reduced total surface water exposure of the molecule. Conversely, this "closed" structure is not present in (−)-epicatechin gallate, which possesses a conformational open and flexible structure.

In addition to the type of protein, pH, and concentration, several structural factors must be taken in account when tannin−protein interactions are analyzed. The influence of procyanidin structural features such as the interflavanoid bond linkage, monomeric units, and esterification with gallic acid is reported herein. This interesting result confirms the greater ability of procyanidins to bind PRPs. Low MW phenolic compounds

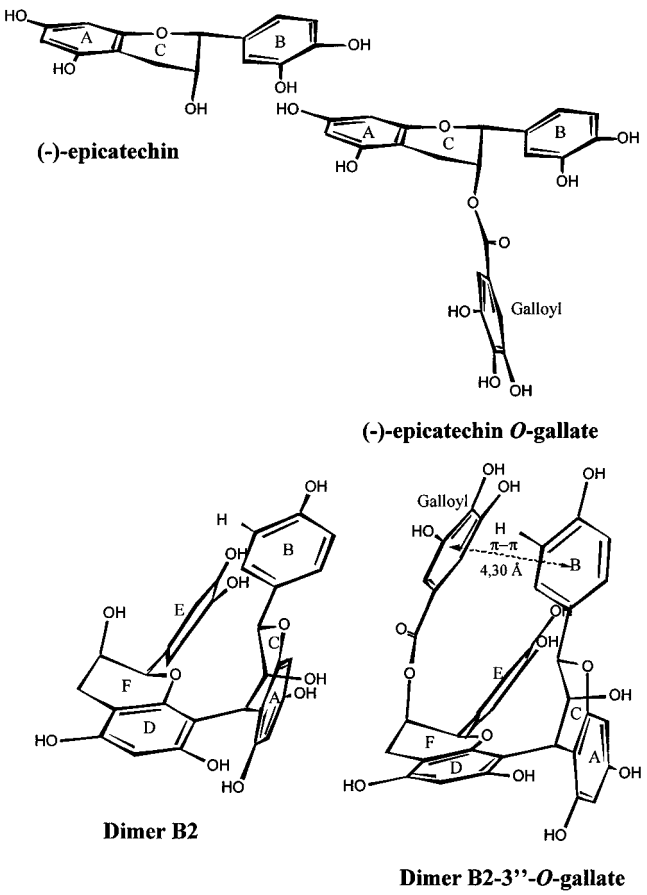

Figure 5. Preferred conformation of (−)-epicatechin, (−)-epicatechin *O*-gallate, dimer B2, and dimer B2−3″-*O*-gallate, determined using Allinger's MM2* force field parameters (*45*).

seem to be too small to effectively cross-link globular proteins. The polyphenols should present sufficient size and adequate composition to be able to bind simultaneously more than one site of the protein surface, acting as a polydentate ligand.

The associations between proteins and procyanidins responsible for the phenomenon of astringency are still difficult to assess. Therefore, further studies involving other procyanidin compounds should be done to bring more insights on this matter for a better understanding of the tannin−protein interactions.

ABBREVIATIONS USED

BSA, bovine serum albumin; NTU, nephelos turbidity units; PRP, proline-rich protein; TSA, tannin specific activity.

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