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Interaction of Different Polyphenols with Bovine Serum Albumin (BSA) and Human Salivary α-Amylase (HSA) by Fluorescence Quenching

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Phenolic compounds are responsible for major organoleptic characteristics of plant-derived food and beverages; these substances have received much attention, given that the major function of these compounds is their antioxidant ability. In the context of this study, our major aim was study the binding of several phenolic compounds such as (+)-catechin, (−)-epicatechin, (−)-epicatechin gallate, malvidin-3-glucoside, tannic acid, procyanidin B4, procyanidin B2 gallate, and procyanidin oligomers to different proteins (bovine serum albumin and human α-amylase) by fluorescence quenching of protein intrinsic fluorescence. From the spectra obtained, the Stern−Volmer, the apparent static, and the bimolecular quenching constants were calculated. The structure of polyphenols revealed to significantly affect the binding/quenching process; in general, the binding affinity increased with the molecular weight of polyphenol compounds and in the presence of galloyl groups. For catechin monomer and procyanidin dimer B4, the K_{SV} was 14100 and 13800 M^{-1}, respectively, and for galloyl derivatives, the K_{SV} was 19500 and 21900 M^{-1}, respectively. Tannic acid was shown to be the major quenching molecule for both proteins. However, comparing different proteins, the same polyphenol showed different quenching effects, which are suggested to be related to the three-dimensional structure of the proteins studied. For (+)-catechin and BSA, the K_{SV} was 8700 M^{-1}, and with α-amylase, it was 14100 M^{-1}; for tannic acid, the K_{SV} was 100548 and 110674 M^{-1}, respectively. From the results obtained, besides the main binding analysis performed, we conclude that this technique is more sensitive than thought because we can detect several interactions that have not been proven by other methods, namely, nephelometry. Overall, fluorescence quenching has proven to be a very sensitive technique with many potentialities to analyze the interaction between polyphenols and proteins.

KEYWORDS: Polyphenols; bovine serum albumin; human salivary α-amylase; fluorescence quenching

INTRODUCTION

Phenolic compounds are responsible for major organoleptic characteristics of plant-derived foods and beverages (color and taste) (*1*). Much attention has been paid to these compounds mainly due to some of their biological properties, given that the major function of these compounds is their antioxidant ability. Indeed, they have been known to induce antimutagenic and anticancerogenic effects (*2*), but many studies have also demonstrated their harmful effects especially when applied in high concentrations (e.g., inhibition of digestive enzymes, decrease in body weight gain, and growth retardation) (*3*, *4*).

Dietary polyphenols show a great diversity of structures, ranging from rather simple molecules, with molecular weights of about 100 Da, to polymers of thousands of Da. Tannins are, in general, complex polyphenols, which are usually 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. Tannins are able to precipitate proteins, thereby being at the origin of their astringent taste. The astringency phenomenon is thought to be due to the interaction of salivary proteins with polyphenols. This event will result in insoluble aggregates that precipitate, reducing the palate lubrification and causing an unpleasant sensation of roughness, dryness, and constriction (*5*, *6*).

Human whole saliva is a complex mixture of some 330 proteins and peptides, without significant differences between men and women (*7*). One of the most important group known to bind phenolic compounds includes salivary proline-rich proteins (PRP; more than 22 proteins are known; proline content 28−40% (*8*, *9*)), which constitute 70% of the proteins in saliva. α-Amylase is also an abundant protein in saliva (∼30% of total proteins) and corresponds to the major enzyme identified in saliva (approximately 58−62 kDa) (*7*).

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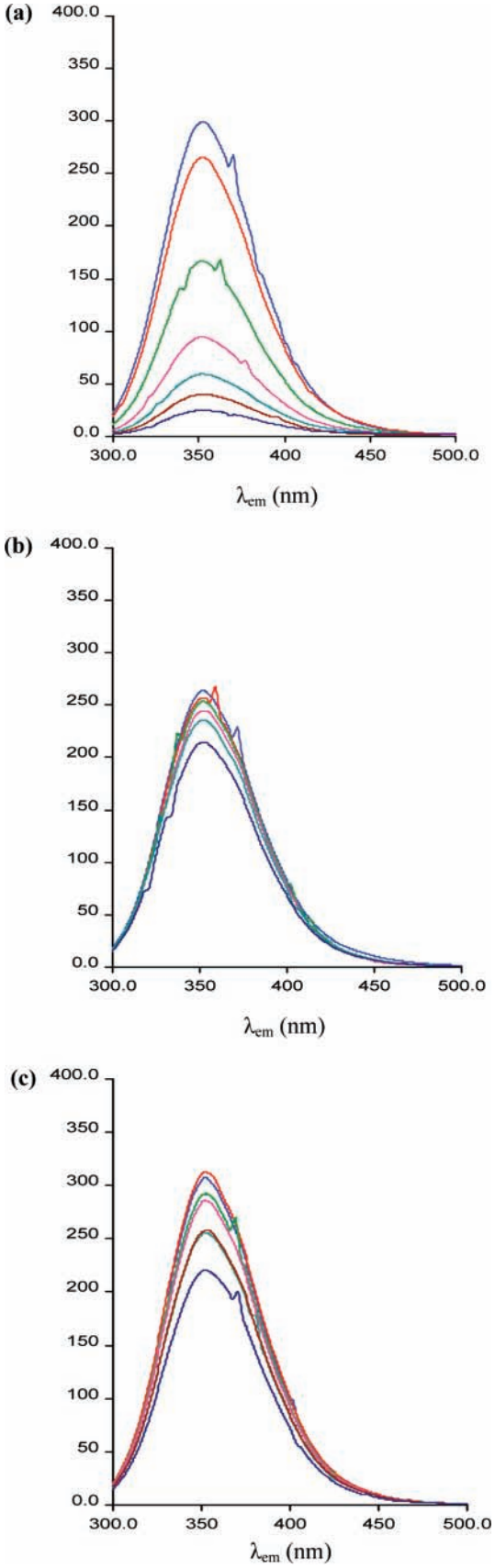

Figure 1. Emission spectra of BSA (3 μM) at λ_{ex} = 282 nm (pH 5.0) in the presence of different concentration of (**a**) tannic acid and (**b**) (+)-catechin; concentration (μM): 0.0 blue, 1.0 red, 5.0 green, 10.0 pink, 15.0 yellow, 20.0 purple, 25.0 indigo. (**c**) Malvidin-3-glucoside; concentration (μM): 0.0 blue, 1.0 red, 5.0 green, 10.0 pink, 15.0 yellow, 20.0 purple, 25.0 indigo.

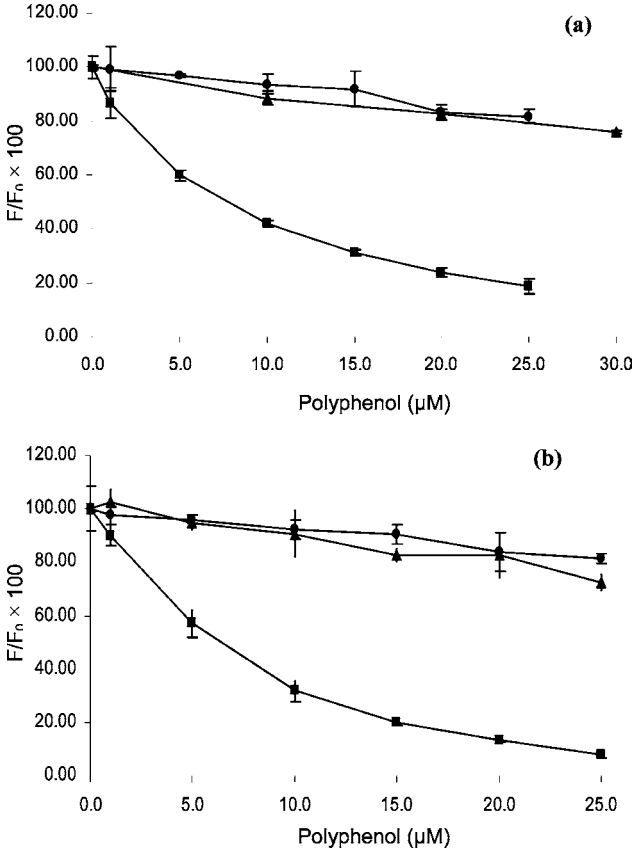

Figure 2. Tryptophan fluorescence quenching of BSA (3 μM) at pH 4.0 (**a**) and pH 5.0 (**b**), plotted as extinction of BSA tryptophans ($F/F_0 \times 100$) against polyphenol concentration for ● (+)-catechin, ▲ malvidin-3-glucoside, and ■ tannic acid. The fluorescence emission intensity was recorded at λ_{ex} = 282 nm, and the λ_{em} maximum occurred at 350 nm. All data were corrected for quencher fluorescence.

Little is known at the molecular level about the interactions of proanthocyanidins with proteins. Direct determination of the composition of tannin−protein complexes is not possible because tannin interferes with conventional assays for protein (*10*). Separation of unbound proanthocyanidins from complexes cannot be accomplished by dialysis because of the polymeric nature of tannins or by gel filtration because of the affinity of phenolic compounds to normally inert chromatographic supports (*11*).

Polyphenol complexation with proteins has been largely studied in solution by NMR spectroscopy, microcalorimetry, enzyme inhibition, protein precipitation, front analysis capillary electrophoresis, turbidity, and nephelometry (*12*−*17*). In the last years, a new technique based on fluorescence quenching has been used by few research groups with the same purpose (*7*, *18*−*20*). In general, existing methods for assaying phenol binding to proteins are labor-intensive, time-consuming, or may require compound-specific assays (*21*−*22*). Unless identification of the site specificity of phenol binding to a protein is desired, quenching of the tryptophan fluorescence of proteins by titration with a ligand may prove to be a rapid and facile method for determining the binding affinities of the phenolic compounds to proteins. This method can also be applied to study interactions of complex food matrices (e.g., tea and wine) with complex protein mixtures in a biological matrix (e.g., human saliva) (*7*).

Fluorescence quenching is the decrease of the quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions with a quencher molecule. Fluorescence quenching can be dynamic, resulting from collisional encounters

between the fluorophore and the quencher, or static, resulting from the formation of a ground-state complex between the fluorophore (protein) and the quencher. In both cases, molecular contact is required between the fluorophore and the quencher for fluorescence quenching to occur (*23*).

There have been several studies on fluorescence quenching of proteins induced by drugs or other small molecules. The interest of this work was directed toward the study by fluorescence spectroscopy of interactions of some phenolic compounds with BSA, a globular protein often used as a model protein to study polyphenol/protein interactions (*7*, *12*−*15*, *18*, *25*), and α-amylase isolated from human saliva.

MATERIALS AND METHODS

Reagents. BSA (≥96%) and (+)-catechin (≥98%) were purchased from Sigma. Tannic acid was purchased from Fluka Biochemica.

α-Amylase purification. α-Amylase was isolated and purified as described previously (*26*, *27*). Briefly, α-amylase was isolated from human saliva after precipitation with ammonium sulfate and chromatography in a DEAE-Sephadex A50 column. The unbound eluate was dialyzed, freeze-dried, and subjected to polyacrylamide gel electrophoresis. The electrophoretic pattern showed that this fraction is rich in α-amylase, with negligible amounts of other proteins. α-Amylase was stocked in a concentration of 7.8 μM and aliquoted in microtubes.

Grape Seed Tannins Isolation. Condensed tannins were extracted as described in the literature (*28*). 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. Then, the 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 HW-40(s) gel column (100 mm × 10 mm i.d., with 0.8 mL min^{-1} of methanol as the eluent), yielding five fractions. fractions I to III were obtained after elution with 99.8% (v/v) methanol for 1/2, 1, and the next 5 h, respectively; fraction IV was obtained after elution with 5% (v/v) methanol/acetic acid for the next 14 h, and fraction V was obtained after elution with methanol/acetic acid, 10% (v/v), for 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 procyanidin composition of each fraction was determined by direct analysis by ESI-mass spectrometry (Finnigan DECA XP PLUS). Fraction I contained catechin, galloyl monomer, and procyanidin dimers (mean MW = 513); fraction II contained procyanidin dimers and galloyl derivatives and procyanidin trimers (mean MW = 823); fraction III contained procyanidin trimers and tetramers and their galloyl derivatives (mean MW = 949); fraction IV contained procyanidin pentamers and also galloyl derivatives (mean MW = 1513); and fraction V contained procyanidin pentamers digalloylated, procyanidin tetramers tetragalloylated, procyanidin hexamers galloylated, procyanidin heptamers, and the galloyl derivatives (mean MW = 2052). The mean molecular weight was determined based on the relative abundance of flavanols in each fraction. The purity's fractions were assessed by HPLC-MS and direct MS analysis, and were higher than 99%.

Isolation of Pure Polyphenols. (-)-Epicatechin gallate, procyanidin B4, and procyanidin B2 gallate were isolated from the fraction I of the grape seed extract purified on Toyopearl gel column and purified by HPLC semipreparative according to the experimental conditions described elsewhere (*28*).

Malvidin-3-glucoside was isolated from a grape skin extract applied to a TSK Toyopearl HW-40(S) gel column (300 mm × 25 mm). After removing the polysaccharides with water, elution was performed with 10% (v/v) methanol/acetic. The solvent was then eliminated using a rotary evaporator at 40 °C. The resulting residue was applied to a C18 gel column, and malvidin-3-glucoside was eluted with 10% methanol acidulated. Again, the solution was concentrated using a rotary evaporator at 40 °C, and the resulting solution was mixed with deionized water, frozen, and lyophilized. The compound's purity was assessed by HPLC-MS and direct MS analysis and was higher than 99%.

Fluorescence Quenching. A Perkin-Elmer LS 45 fluorimeter was used for fluorescence quenching measurements. The excitation wavelength was set at 282 nm, and the emission was recorded from 200 to 800 nm. Both slits were 10 nm.

The experiments were performed in two different solvents: solvent A, water/DMSO (9:1, v/v), 50 mM citrate buffer, pH 4,0; and solvent B, ethanol/water (1.2:8.8, v/v), 100 mM acetate buffer, pH 5.0. The pH of 4.0 and 5.0 were chosen because it is already known that BSA and α-amylase interactions with polyphenols are greater at these pH, respectively. Small quantities of DMSO are often used to solubilize polyphenols in aqueous solvents (*16*).

Stock solutions of BSA (3 μM) and polyphenols (100 μM) such as (+)-catechin, tannic acid and malvidin-3-glucoside were prepared in solvents A and B. Stock solutions of α-amylase (3 μM) and polyphenols (100 μM) such as (−)-epicatechin gallate, procyanidin B4, procyanidin B2 gallate, and fractions I−V were prepared in solvent B. All solutions were carefully filtered (0.45 μm). The concentration of proteins (3 μM) was in the linear range of concentration with fluorescence.

In several 2 mL microtubes, BSA solution (200 μL) was mixed with different volumes of polyphenol stock solutions (from 0 to 50 μL) in order to give final concentrations of polyphenols in the range of 0−25 μM. The tubes were vortexed for 10 s. After this, the solutions were transferred to the fluorimeter cell where the emission spectra were measured. In α-amylase experiments, only solvent B was studied.

Between each measurement, the cell was washed three times with water. Because some polyphenols possess intrinsic fluorescence at the λ_{ex} (*16*), in all experiments, a blank was made for each polyphenol concentration, in which protein solution was replaced only by solvent A or B. The blank spectrum was automatically subtracted from the emission spectrum of the corresponding solution. All experiments were performed in triplicate, and the mean values were calculated.

Principles of Fluorescence Quenching (*23*). Fluorescence quenching is described by the Stern−Volmer equation (eq 1)

$$\frac{F_0}{F} 1 + k_q \tau_0 [Q] = 1 + K_{SV}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities before and after the addition of the quencher (polyphenol), respectively, k_q is the bimolecular quenching constant, τ_0 is the lifetime of the fluorophore in the absence of the quencher, $[Q]$ is the concentration of the quencher, and K_{SV} is the Stern−Volmer quenching constant. Hence, eq 1 was applied to determine K_{SV} by linear regression of a plot of F_0/F against $[Q]$.

A linear Stern−Volmer plot is generally indicative of a single class of fluorophores in a protein, all equally accessible to the quencher; this also means that only one mechanism (dynamic or static) of quenching occurs. In the cases of a static mechanism, there is a complex formation, and in such cases, the bimolecular quenching constant is calculated; k_q can be calculated by the ratio between K_{SV} and τ_0. For BSA, the lifetime of the fluorophore is approximately 5 ns (*18*), and for α-amylase, the lifetime of the fluorophore is approximately 2.97 ns (*29*). The maximum value possible for diffusion-limited quenching (dynamic mechanism) in water is $\sim 10^{10}$ M^{-1} s^{-1}. When the value of the bimolecular quenching constant is higher, it could mean that there is a complex formation between protein and quencher, corresponding to a static mechanism. There have been several studies reporting BSA quenching due to specific interactions (*30*), and in these cases, the quenching constant has been much higher than the maximum value of diffusion-limited quenching in water. Smaller values of k_q can result from steric shielding of the fluorophore.

However, positive deviations from the Stern−Volmer equation are frequently observed when the extent of quenching is large. In that case, the Stern−Volmer plot exhibits an upward curvature, concave toward the y axis at high $[Q]$. These positive deviations may be an indication of two distinct situations. In many cases, this upward curvature indicates that the fluorophore can be quenched by both mechanisms with the same quencher. In other cases, the upward curvature indicates the presence of a sphere of action. This assumes the existence of a sphere

of volume around a fluorophore within which a quencher will cause quenching with a probability of unity. In this situation, quenching occurs due to the quencher being adjacent to the fluorophore at the moment of excitation. These closely spaced fluorophore−quencher pairs are immediately quenched, but fluorophores and quenchers do not actually form a ground-state complex. This type of apparent static quenching is usually interpreted in terms of the model "sphere of action". The modified form of the Stern−Volmer equation which describes this situation is

$$\frac{F_0}{F} = (1 + K[Q])\exp([Q]VN/1000)$$

In this equation, V is the volume of the sphere, and N is the Avogadro's constant. If $K[Q]$ is small enough, $(1 + K[Q]) \approx \exp(K[Q])$, which is equivalent to $\exp([Q]VN)$ (*31*). Thus, the preview equation becomes eq 2

$$\frac{F_0}{F} = e^{(K[Q])} \quad (2)$$

Statistical Analysis. All assays were performed in $n = 3$ repetitions. The mean values, standard deviations, and statistical differences were evaluated using analysis of variance (ANOVA); the mean values were compared using a Turkey test, and all statistic data were processed using the Origin Pro Software (Origin Lab, Corporation, Massachusetts).

RESULTS AND DISCUSSION

The conformational changes of BSA and α-amylase were evaluated by the measurement of the intrinsic fluorescence intensity of protein tryptophan residues before and after the addition of polyphenols. Fluorescence measurements give information about the molecular environment in the vicinity of the chromophore molecule. Changes in the emission spectra of tryptophan are common in response to protein conformational transitions, subunit association, substrate binding, or denaturation.

Effect of Flavonoids on BSA Spectra. Figure 1 shows the fluorescence emission spectra ($\lambda_{ex} = 282$ nm) obtained for BSA at pH 5.0 with the addition of (+)-catechin, malvidin-3-glucose, and tannic acid. In all cases, a decrease in the fluorescence intensity caused by quenching was observed, but there was no shift of the maximum λ_{em}. The same behavior was observed at pH 4.0 (data not shown).

All of the spectra presented were automatically corrected, subtracting the spectra of the respective polyphenol. This mode of correction has already been validated (*18*).

Fluorescence Quenching of BSA. Figure 2 shows the raw data for quenching of BSA fluorescence at pH 4.0 and 5.0 by addition of (+)-catechin, malvidin-3-glucoside, and tannic acid.

These results indicate that the changes of the environment of tryptophan residues depend on the polyphenolic structure, being very similar at both pHs studied. The experiment with tannic acid was found to eventually lead to total quenching, while (+)-catechin and malvidin-3-glucoside quenched only 20−30%. Both (+)-catechin and malvidin-3-glucoside had a similar effect on BSA fluorescence; the extinction of BSA tryptophans decreased slowly and almost linearly (**Figure 2**) but did not reach zero over the range of the concentrations studied. Opposite to tannic acid, it is well-known that (+)-catechin and malvidin-3-glucoside (low molecular weight polyphenols) do not have a good affinity to complex with proteins, which agrees with these results (*17*, *24*, *34*, *35*).

Figure 3 shows the Stern−Volmer plots for the BSA fluorescence quenching by (+)-catechin and malvidin-3-glucoside. **Table 1** summarizes the calculated K_{SV} and the bimolecular

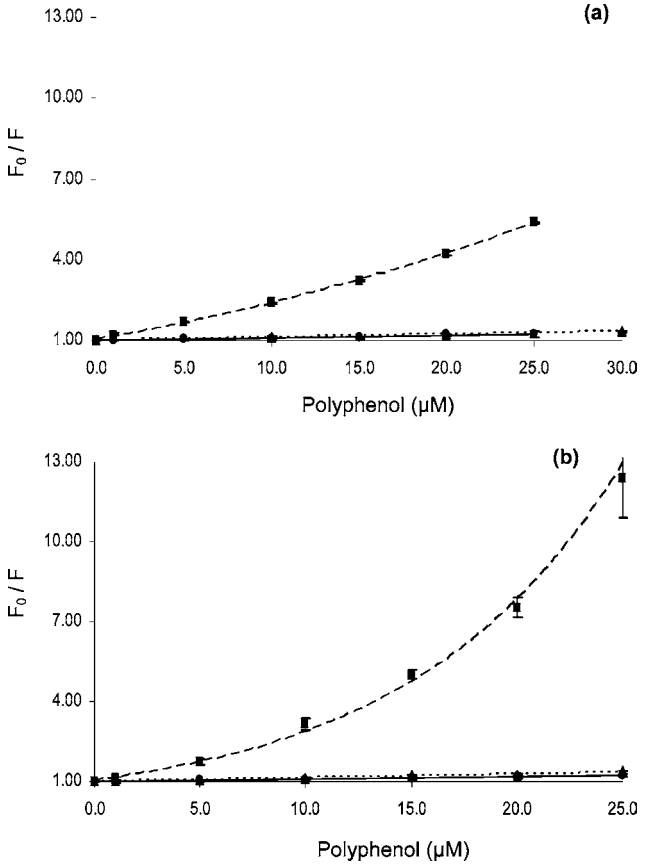

Figure 3. Stern–Volmer plot describing tryptophan quenching of BSA (3 μM) at pH 4.0 (**a**) and pH 5.0 (**b**) in the presence of different concentrations of polyphenols. (**a**) ● (+)-catechin, $y = 0.0091x + 0.9896$; ▲ malvidin-3-glucoside, $y = 0.0105x + 1.0086$; ■ tannic acid, $y = 0.0026x^2 + 0.1082x + 1.0314$. (**b**) ● (+)-catechin, $y = 0.0087x + 1.0006$; ▲ malvidin-3-glucoside, $y = 0.0147x + 0.9742$; ■ tannic acid, $y = 1.0476e^{0.1005x}$. Fluorescence emission intensity was recorded at $\lambda_{ex} = 282$ nm, and the λ_{em} maximum occurred at 350 nm.

quenching constant at pH 4.0 and 5.0. The constants obtained at different pHs were very similar. Malvidin-3-glucoside has shown to be slightly more reactive toward quenching BSA fluorescence than (+)-catechin, especially at pH 5.0.

The K_{SV} for (+)-catechin was not in agreement with previous results (**Figure 3**). Papadopoulou et al. (*18*) have found a different result (16500 M^{-1} for pH 5.0) using the same technique but with different experimental conditions (solvent composition and protein concentration).

To interpret the data from fluorescence quenching studies, it is important to understand what kind of interaction occurs between the quencher and the fluorophore. In this case, the Stern−Volmer plots for (+)-catechin and malvidin-3-glucoside are linear, which means that only one type of quenching mechanism occurs (dynamic or static) (*18*).

The bimolecular quenching constant (k_q) allows one to verify if the quenching is due to a complex formation with proteins that affect tryptophan's microenvironment. The bimolecular quenching constant can reflect the efficiency of quenching or the accessibility of the fluorophores to the quencher. The dynamic mechanism (diffusion-controlled quenching) typically results in values of k_q near 1×10^{10} M^{-1} s^{-1} (*23*). The k_q of (+)-catechin and malvidin-3-glucoside is 200-fold higher, which suggests a complex formation between BSA and these polyphenols (static mechanism). There have been studies reporting BSA quenching by a specific interaction with tannic acid and

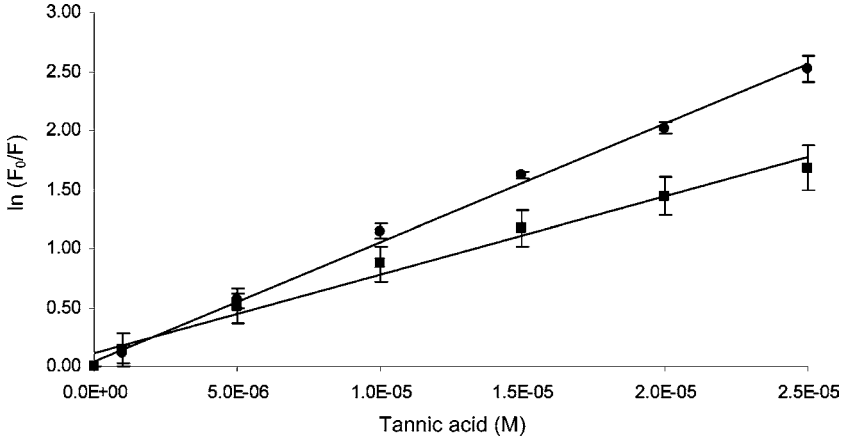

Figure 4. Modified form of a Stern–Volmer plot for tannic acid at ■ pH 4.0, $y = 66512x + 0.1087$; and ● pH 5.0, $y = 100548x + 0.0465$, based on eq 2.

Table 1. Stern–Volmer (K_{SV}) and Bimolecular (k_q) Quenching Constants, for the Interaction of Two Polyphenols with BSA, for Two pHs. Values with Different Superscript Letters Are Significantly Different ($P < 0.05$); Values with the Same Superscript Letters Are Not Significantly Different ($P > 0.05$)

	pH 4.0		pH 5.0	
polyphenol	K_{SV} (M^{-1})	$k_q \times 10^{12}$ (M^{-1} s^{-1})	K_{SV} (M^{-1})	$k_q \times 10^{12}$ (M^{-1} s^{-1})
(+)-catechin	9100 ± 265^a	1.82 ± 0.05^d	8700 ± 1054^a	1.74 ± 0.21^d
malvidin-3-glucoside	10500 ± 404^b	2.10 ± 0.08^e	14700 ± 557^c	2.94 ± 0.11^f

Table 2. Apparent Static (K) and Bimolecular (k_q) Quenching Constants for the Interaction of Tannic Acid with BSA at Two pHs. Values with Different Superscript Letters Are Significantly Different ($P < 0.05$); Values with the Same Superscript Letters Are Not Significantly Different ($P > 0.05$)

	K (M^{-1})	$k_q \times 10^{12}$ (M^{-1} s^{-1})
pH 4.0	66512 ± 1942^a	13.30 ± 0.39^c
pH 5.0	100548 ± 1777^b	20.11 ± 0.36^d

derivatives (ellagic and gallic acids) (*19*, *30*), and in these cases, the quenching constant has been also several magnitudes higher than the maximum value of diffusion-limited quenching in water.

Relative to tannic acid, the Stern−Volmer plot presented for both pHs has an upward curvature, concave toward the *y* axis (**Figure 3**). As previously mentioned, this characteristic feature could mean that the protein can be quenched by both mechanisms, or it could mean the existence of a "sphere of action". In this case, the quenching of BSA fluorescence by tannic acid at both pHs obeys the modified form of the Stern−Volmer equation (eq 2) described by the "sphere of action model" (**Figure 4** and **Table 2**).

The apparent static quenching constant (K) is lower at pH 4.0 than it is at pH 5.0, which reflects the higher affinity of tannic acid for BSA at pH 5.0. The apparent static quenching constant of tannic acid is much higher than the ones obtained for (+)-catechin and malvidin-3-glucoside, which reflects the higher affinity of tannic acid to bind BSA.

Bovine serum albumin (BSA) consists of 582 amino acid residues forming a single polypeptide, organized in three homologous α-helix domains (I−III). Each domain contains ten helices and is divided into antiparallel six helix and four subdomains (A and B). BSA contains two tryptophan residues with intrinsic fluorescence, the first one in position 134 (located on the surface of the molecule) and the second in position 212 (located within a hydrophobic pocket of the protein) (*18*, *32*). Furthermore, these residues are located in separate domains within the BSA molecule (*18*); therefore, these residues are in distinct environments. Each residue can be accessible differently to the quencher.

The dominant fluorophore is the indole group of tryptophan. Indole absorbs near 280 nm and emits near 340 nm. The emission of indole may be blue shifted if the group is buried within a native protein, and its emission may shift to longer wavelengths (red shift) when protein is unfolded (*23*). When considering the effect of flavonoids on the fluorescence spectra of BSA, there was no shift of λ_{em}. This suggests that there was no change in the immediate environment of the tryptophan residues, other than the fact that the polyphenols were situated at close proximity to the tryptophan residue for the quenching to occur. This means that the molecular conformation of the protein was not significantly effected, regardless of the polyphenol mechanism of interaction. This idea is opposite to a recent study that has shown that the tertiary structure of proteins (human serum albumin, bovine serum albumin, soy glycinin, and lysozyme) changes upon binding of phenolic compounds (chlorogenic, ferulic, and gallic acids, quercetin, rutin, and isoquercetin), while the secondary structure remains intact (*21*). However, the experimental conditions and the polyphenols used were not the same.

As mentioned above, BSA has two tryptophan residues in a distinct environment (hydrophobic and hydrophilic environment). The results for (+)-catechin and malvidin-3-glucoside show that tryptophan residues are effected equally, resulting in linear Stern−Volmer plots. This suggests that these polyphenols interact simultaneously with the interior hydrophobic pockets of BSA and with the hydrophilic surface of the protein (*18*). This latter mode of association agrees with earlier results where isothermal titration calorimetry was employed for the investigation of protein−tannin interactions (*13*).

Some research showed that tannic acid (hydrolyzable tannin containing eight galloyl groups) has an unusually high binding affinity for proteins (*17*, *19*, *41*). Other studies have shown that

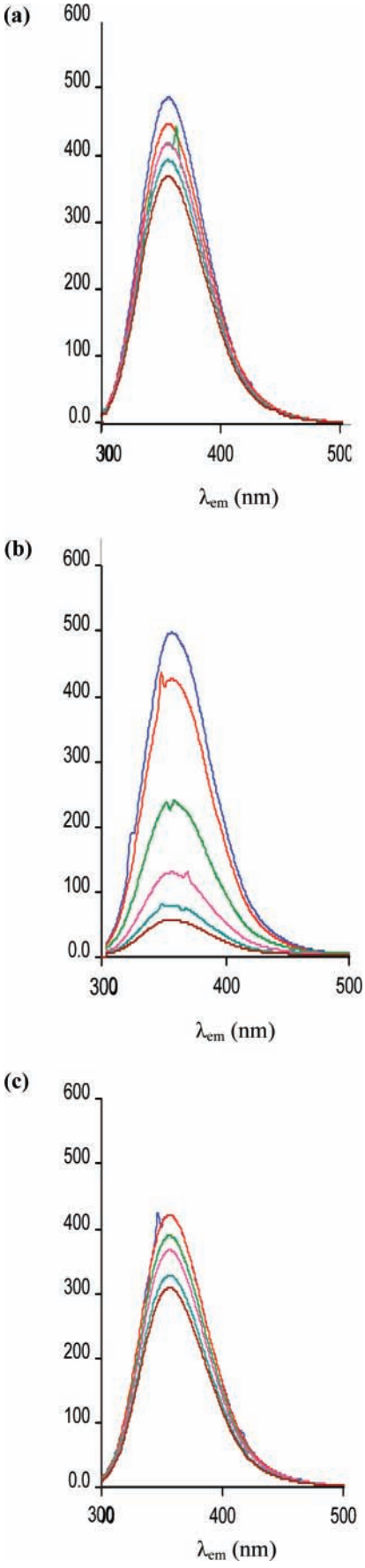

Figure 5. Emission spectra of α-amylase (3 μM) at λ_{ex} = 282 nm (pH 5.0) in the presence of different concentrations of (**a**) catechin, (**b**), tannic acid, and (**c**), (–)-epicatechin gallate; concentration (μM): 0.0 blue, 1.0 red, 5.0 green, 10.0 pink, 15.0 yellow, 20.0 purple.

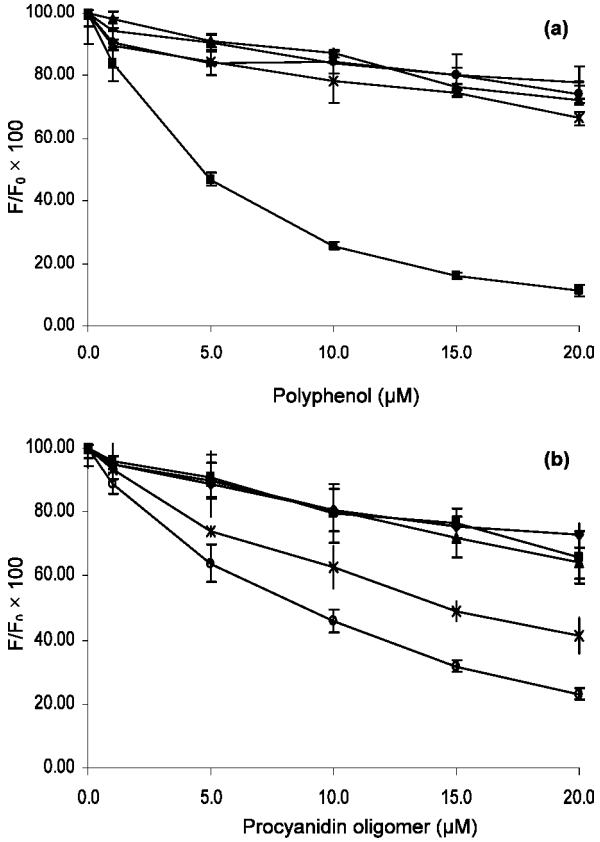

Figure 6. Tryptophan fluorescence quenching of α-amilase (3 μM) at pH 5.0, plotted as extinction of α-amilase tryptophans ($F/F_0 \times 100$) against polyphenol concentration for (**a**) ● (+)-catechin, ■ tannic acid, ▲ (–)-epicatechin gallate, ∗ procyanidin B2 gallate, and — procyanidin B4; and procyanidin oligomer concentration for (**b**) ◆ fraction I (MW = 513), ■ fraction II (MW = 823), ▲ fraction III (MW = 949), × fraction IV (MW = 1513), and *o* fraction V (MW = 2052). Fluorescence emission intensity was recorded at λ_{ex} = 282 nm, and the λ_{em} maximum occurred at 355 nm (**a**) and 350 nm (**b**). All data were corrected for quencher fluorescence.

the contribution of the galloyl groups is important; compounds containing galloyl groups have more binding activity toward proteins (*34*). This explains why tannic acid has an apparent static quenching constant (*K*) that is so high. Each galloyl group thereby provides three hydroxyl groups and a benzene ring, which can establish hydrogen and hydrophobic bonds, respectively, increasing the binding affinity to BSA. The great affinity of polyphenol groups to complex proteins resulting from hydrogen and hydrophobic bonds probably justifies the prevalence of the static mechanism in comparison to the dynamic one. It is also evident that pH influences the binding affinity. The increase of pH from 4.0 to 5.0 leads to an increase in the charge of tannic acid (although not significantly) (*26*), and the pH becomes close to the BSA isoelectric point (pI) (4.7). Therefore, at pH 4.0, BSA has a positive charge, and at pH 5.0, it has a negative charge. The variation in tannic acid and BSA charges could be responsible for the variation in the apparent static constant.

Effect of Flavonoids on α-Amylase Spectra. The effect of several polyphenols ((+)-catechin, (−)-epicatechin-gallate, and tannic acid) on the α-amylase fluorescence quenching was also studied. **Figure 5** shows the fluorescence emission spectra (λ_{ex} = 282 nm) obtained for α-amylase at pH 5.0 with the addition of (+)-catechin, (−)-epicatechin-gallate, and tannic acid. Once again, in all cases, a decrease in the fluorescence intensity caused by quenching was observed, but there was no shift of the

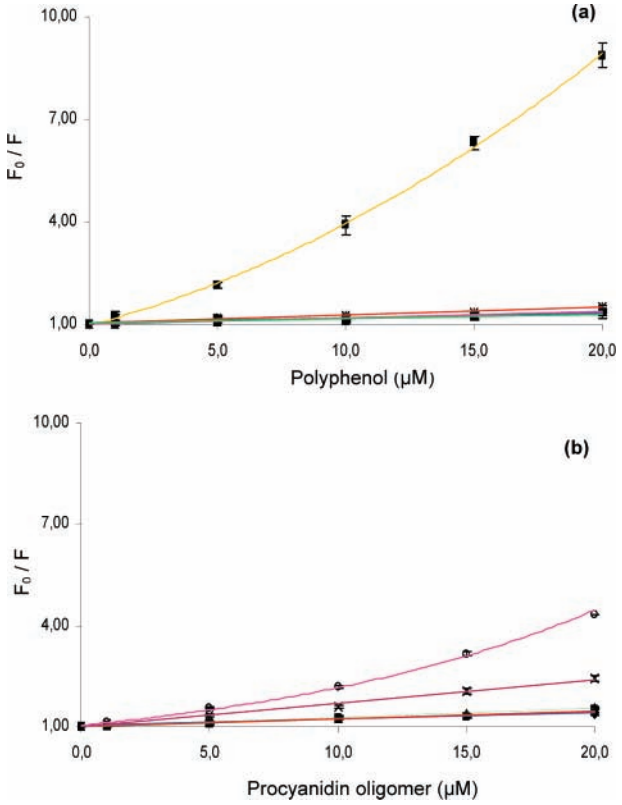

Figure 7. Stern–Volmer plot describing tryptophan quenching of α-amilase (3 μM) at pH 5.0 caused by polyphenol association. (**a**) ■ Tannic acid, $y = 0.0102x^2 + 0.2009x + 0.9788$ (yellow); ● (+)-catechin, $y = 0.0141x + 1.0598$ (blue); ▲ (–)-epicatechin gallate, $y = 0.0195x + 0.9941$ (pink); ∗ procyanidin B2 gallate, $y = 0.0219x + 1.0511$ (red); — procyanidin B4, $y = 0.0138x + 1.031$ (green); and by procyanidin oligomers. (**b**) ◆ Fraction I, $y = 0.0187x + 1.0269$ (blue); ■ fraction II, $y = 0.0241x + 0.9981$ (red); ▲ fraction III, $y = 0.0268x + 0.9971$ (green); × fraction IV, $y = 0.0701x + 0.9842$ (purple); *o* fraction V, $y = 1.0441e^{0.0725x}$ (pink). The fluorescence emission intensity was recorded at $\lambda_{ex} = 282$ nm, and the λ_{em} maximum occurred at 355 (**a**) and 350 nm (**b**).

maximum λ_{em}. Similar effects were observed for the procyanidin fractions (data not shown).

Fluorescence Quenching of α-Amylase. Figure 6 shows the data for quenching of α-amylase fluorescence at pH 5.0 by addition of (+)-catechin, tannic acid, (-)-epicatechin gallate, procyanidin B2 gallate, procyanidin B4, and procyanidin oligomers (fractions I−V).

All of the tested compounds were shown to decrease α-amylase fluorescence; the extinction of α-amylase tryptophans decreased slowly and almost linearly (**Figure 6**) until 20−30% quenching. Fractions IV and V and especially tannic acid led to an important clearance of α-amylase fluorescence.

The K_{SV} and k_q obtained directly from the slope of Stern−Volmer plots for all tested compounds which have a linear graphic (**Figure 7**) are summarized in **Table 3**. Fraction IV revealed the highest quenching constant and fraction *I*; (+)-catechin and procyanidin B4 showed the lowest values.

The values obtained for bimolecular quenching constants are all greater than 10^{10} M^{-1} s^{-1} (characteristic of a static mechanism), which suggests the formation of a complex between α-amylase and the polyphenols studied, as previously observed with BSA and (+)-catechin.

The data of the Stern−Volmer plots of fraction V and tannic acid present an upward curvature, concave toward the *y* axis (**Figure 7**). This behavior was also observed for the interaction between tannic acid and BSA. α-Amylase fluorescence quenching by tannic acid and fractions V obeys the modified Stern−Volmer eq 2 (**Figure 8**), characteristic of the "sphere of action" model.

Table 3. Stern–Volmer (K_{SV}) and Bimolecular (k_q) Quenching Constants for the Interaction of Polyphenols with α-Amylase at pH 5.0. Values with Different Superscript Letters Are Significantly Different ($P < 0.05$); Values with the Same Superscript Letters Are Not Significantly Different ($P > 0.05$)

flavonoid		K_{SV} (M^{-1})	$k_q \times 10^{12}$ (M^{-1} s^{-1})
(+)-catechin		$14100 \pm 3656^{a,b}$	$4.75 \pm 1.23^{f,g}$
(–)-epicatechin-gallate		19500 ± 500^{c}	6.53 ± 0.17^{h}
procyanidin B2 gallate		21900 ± 1976^{c}	7.37 ± 0.67^{h}
procyanidin B4		13800 ± 1387^{a}	4.78 ± 0.47^{f}
procyanidin oligomers	I	$18700 \pm 1528^{b,c}$	$6.29 \pm 0.51^{g,h}$
	III	$24100 \pm 2615^{c,d}$	$8.08 \pm 0.88^{h,i}$
	III	26800 ± 1447^{d}	8.98 ± 0.49^{i}
	IV	70100 ± 2593^{e}	23.67 ± 0.8^{j}

Table 4 summarizes the calculated constants from the modified Stern−Volmer plot (eq 2), which reflect the high affinity of tannic acid (as also observed for BSA) and fraction V for α-amylase. The apparent static quenching constant obtained for tannic acid is much higher than the one obtained for fraction V.

Human α-amylase (HSA) is a secretory protein that is produced by both the pancreas and salivary glands. HSA consists of 496 amino acids and is found in two forms in human saliva, a glycosylated isoform (apparent molecular weight 62 kDa) and a non-glycosylated form of 56 kDa (*33*). This protein contains 22 proline and 16 tryptophan amino acid residues in its sequence.

(+)-Catechin is the polyphenol compound with the lowest molecular weight and smallest structure, being one with the lower binding constant. Procyanidin B4 is a C4−C8 dimer of catechin, and its binding constant was found to be slightly lower than the one of (+)-catechin (statistically, they are not significantly different). Despite the increase of aromatic rings and hydroxyl groups with the increase of catechin units available to form hydrophobic and hydroxyl bonding with α-amylase, (+)-catechin and dimer B4 seem to have the same ability to complex α-amylase. This may be explained by a loss of some conformational mobility of dimer B4 compared to (+)-catechin. However, the binding constant of (−)-epicatechin gallate is higher than that of (+)-catechin and dimer B4. The presence of a galloyl group, composed of an aromatic ring with three hydroxyl groups available to establish hydrophobic and hydrogen bonds with α-amylase, seems to improve the interaction between both. This is also observed for B2 gallate for which the presence of a galloyl group increases the binding constant toward α-amylase significantly compared to that of dimer B4.

Despite the considerable size of tannic acid, its eight galloyl groups are apparently responsible for the higher constant without any conformational hindrance.

The oligomeric procyanidin fractions used were obtained from grape seed extracts. Fraction I (average MW = 513) contained catechins ((+)-catechin, (−)-epicatechin, (−)-epicatechin gallate) and essentially procyanidin dimers. Fraction II (average MW = 823) contained procyanidin dimers and galloyl derivatives and procyanidin trimers, the trimers being the most abundant. Fraction III (average MW = 949) contained procyanidin trimers and tetramers and its galloyl derivatives, the trimers being the most abundant. Fraction IV (average MW = 1513) contained procyanidin pentamers and galloyl derivatives. Frac-

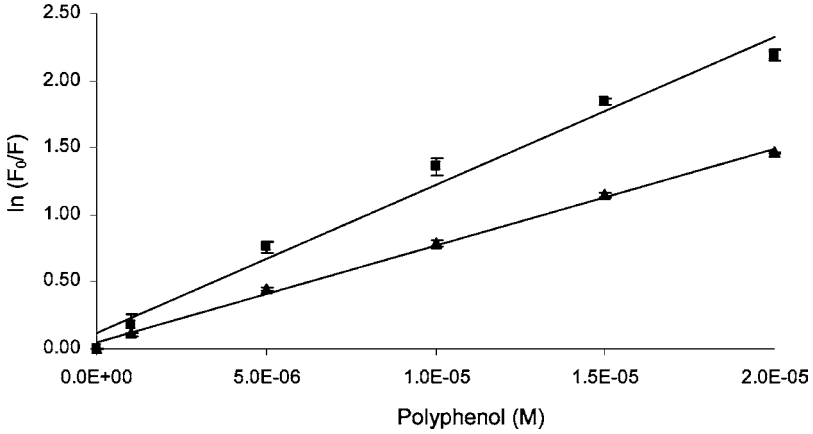

Figure 8. Modified forms of a Stern–Volmer plot for ■ tannic acid, $y = 110674x + 0.1127$, and ▲ fraction *V*, $y = 72515x + 0.0432$, based on eq 2.

Table 4. Apparent Static (*K*) and Bimolecular (k_q) Quenching Constants for the Interaction of Tannic Acid and Fraction V with α-Amylase at pH 5.0. Values with Different Superscript Letters Are Significantly Different ($P < 0.05$); Values with the Same Superscript Letters Are Not Significantly Different ($P > 0.05$)

compound	K (M^{-1})	$k_q \times 10^{12}$ (M^{-1} s^{-1})
tannic acid	110674 ± 103^a	37.26 ± 0.04^c
fraction V	72515 ± 399^b	24.42 ± 0.13^d

Table 5. Amino Acid Residues Composition of BSA and α-Amylase

	number of amino acid residues in each protein	
amino acid	BSA (40)	α-amylase
proline (P)	28	22
histidine (H)	17	12
arginine (R)	26	28
phenylalanine (F)	30	26
tryptophan (W)	2	17

tion V (average MW = 2052) contained procyanidin pentamer digallates, procyanidin tetramer tetragallates, procyanidin hexamer gallates, the procyanidin heptamer. and its gallate derivatives. Resuming, the average molecular weight increased along the fractions I−V.

The Stern−Volmer constant increased with the procyanidin molecular weight. However, the constants of fractions I, II, and III showed a similar magnitude (statistically, they are not significantly different); the highest fractions IV and V had much higher constants.

The number of catechin units and galloyl groups increased with the molecular weight of the procyanidin oligomers. This resulted in a higher number of hydrophobic sites (phenolic rings and hydroxyl groups) available to interact with protein binding sites. Therefore, a stronger binding affinity was anticipated for the high molecular weight procyanidin oligomers, especially fractions IV and V. The increase of the binding affinity of grape seed procyanidins to proteins with the rise of molecular weight was also reported in the literature by different methods (*34*−*36*).

Previous studies indicated that condensed tannins are more effective at precipitating proteins than hydrolyzable tannins (*37*, *38*). The specificity of tannin−protein interactions is believed to be a function of the size, conformation, and charge of both tannin and protein molecules. These ideas are in agreement with our results, if assuming that the Stern−Volmer quenching constant corresponds to a binding constant.

The study of the interaction between α-amylase and BSA and some polyphenol compounds (namely, (+)-catechin, (−)-epicatechin gallate, and procyanidin dimers and trimers) has been previously performed using a nephelometric technique (*39*), but this study did not detect any aggregation between them, probably due to a lack of sensitivity. Interestingly, the method described herein allows one to verify that interaction between polyphenolic compounds and the proteins does occur. Thus, fluorescence quenching has proven to be a very sensitive technique, with many potentialities to analyze the interaction between polyphenols and proteins.

Comparison of Fluorescence Quenching of BSA and α-Amylase. Early studies of polyphenol/protein binding suggested that polyphenols bind preferentially to proline residues. However, proline is certainly not the only possible binding site. For example, it has been suggested that polyphenols bind tightly to histatins, which are salivary proteins containing a high proportion of histidine residues. Proline is an important binding site, but interactions also occur with arginine and phenylalanine side chains. However, it should be noted that, in most proteins, phenylalanine side chains are buried within the protein structure and therefore are not, in general, available as binding sites. Arginine can strengthen the interaction between polyphenols and peptides by forming an additional site of interaction but does not by itself form an independent strong binding site. Following these principles, it is tempting to assume that the differences between the binding of the same polyphenol to different proteins result from differences in the amino acid sequences.

However, after a detailed analysis of the previously discussed amino acids involved in polyphenol binding, we can conclude that differences between the number and spatial distribution of amino acids in the two proteins tested in this work are not significant (**Table 5**).

Both proteins analyzed (BSA and α-amylase) are globular and have similar sizes (66 kDa for BSA and 56 kDa for amylase); although the global structure is similar, there are small differences that are crucial to the different binding abilities. As well, the three-dimensional distribution of the discussed amino acid residues in both proteins is not significantly different.

Analyzing the three-dimensional structure of BSA, a large pocket can be observed (*40*), which is related to the function of this protein (carrier molecule). This cavity could allow the accommodation of a ligand with considerable size (e.g., tannic acid). The proline residues are dispersed along the surface and inside of the cavity of BSA, allowing binding in both regions. Therefore, this cavity could be responsible for the high affinity of tannic acid that would act as a multidentate ligand. In the case of small molecules, like (+)-catechin, they are not likely

to establish many bonds in the large pocket, hence not binding properly like tannic acid.

Relative to α-amylase proteins, they have a multidomain structure, being more compacted proteins. The cavity formed by various domains is small, which allows a good fit of small molecules (e.g., (+)-catechin). This feature could explain the higher affinity of small compounds for α-amylase relative to that for BSA.

From the ideas presented, (+)-catechin would have more binding affinity for α-amylase than for BSA, which is confirmed by Stern−Volmer quenching constants of 14100 and 8700 M^{-1} (at pH 5.0), respectively. In the case of tannic acid, the situation is not similar, with a small difference between binding affinities of this compound and the two proteins. However, tannic acid showed more affinity for α-amylase than for BSA, with Stern−Volmer quenching constants of 110674 and 100548 M^{-1}, respectively.

This hypothesis is also interesting from a biological perspective. In a recent study (*7*), there was evidence that the interaction of α-amylase with tannins resulted in a decrease of its activity. This feature could occur due to the high affinity between tannins and α-amylase.

Conclusion. The binding affinities of BSA and α-amylase with polyphenols 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 structure of the polyphenols has some effect on the quenching process. The size and structural features, such as the presence of galloyl groups, of the quencher molecule is related to its quenching capacity. For the same protein, the smaller polyphenol compound is the weakest quenching molecule because it is the one that provides fewer binding groups. However, in different proteins, the same polyphenol molecule could have different quenching effects and binding affinities, which are related to the three-dimensional structure of the protein.

The primary reaction leading to the sensation of astringency is the precipitation of salivary proteins by polyphenolic compounds (*41*). Tannic acid was shown to be the major quenching molecule for both proteins and is also known to be a very astringent compound, even more than the other compounds tested. Therefore, the binding constants determined by the fluorescence quenching method could be very helpful for understanding, at a molecular level, the phenomena that could be at the origin of food astringency, which remains a matter of interest and is not well-known.

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