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ARTICLE

Biological Relevance of the Interaction between Procyanidins and Trypsin: A Multitechnique Approach

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The interactions between the digestive protease trypsin type IX-S from porcine pancreas and grape seed procyanidins were monitorized by fluorescence quenching, dynamic light scattering, nephelometry, circular dichroism, and enzymatic inhibition assay. This work reports that the inhibition of trypsin activity by grape seed procyanidins and the respective quenching of intrinsic protein fluorescence are closely related. These two phenomena increase with the molecular weight of the tested procyanidins. The interaction between procyanidins and enzyme was shown to involve a specific interaction as inferred from the fluorescence assays. It was also shown by fluorescence spectroscopy that the binding of procyanidin molecules to the enzyme does not induce significant structural modifications. A relationship between aggregate formation, using dynamic light scattering and nephelometry, and fluorescence quenching was observed with maxima achieved for similar stoichiometric ratios. The binding of procyanidins to trypsin affects only slightly protein structure as seen by circular dichroism.

KEYWORDS: Antinutritional; procyanidin; fluorescence quenching; dynamic light scattering; circular dichroism

INTRODUCTION

The digestive enzyme trypsin (EC 3.4.21.4) occurs in the mammalian intestine and belongs to the group of the serine proteases. The inhibition of this class of enzymes is considered relevant in medicinal chemistry for the treatment of diseases such as platelet aggregation disorders, rheumatoid arthritis, pancreatitis, cystic fibrosis, pulmonary emphysema, and asthma (*1*). On the other hand, a reduction in the activity of serine proteases, in particular trypsin, may contribute to a reduced absorption of nutrients. This enzyme is responsible for the breakdown of proteins into peptides and amino acids that are readily absorbed through the intestinal mucosa (*2*). Trypsin is also responsible for a cascade activation phenomenon in the intestine involving the breakdown of zymogen precursors producing active digestive enzymes of the various classes (*2*). Therefore, trypsin activity inhibition may contribute directly to a reduced digestion of protein and indirectly to reduced digestion of other nutrients via digestive enzyme nonactivation.

Phenolic compounds are abundant vegetable secondary metabolites present in the human diet both in fruits and in beverages (*3*). These compounds have been ascribed a large number of beneficial effects mostly because of their antioxidant properties (*4*) as well as some nutritionally harmful effects (*5*). The latter appear to be in part related to their ability to inhibit enzymatic activity. This capacity has been proven over the last years even though their inhibition of digestive enzymes has only been scarcely studied (*6*, *7*).

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Procyanidins (condensed tannins) are the family of phenolic compounds which possesses the highest ability to complex with proteins. The interaction between procyanidins and proteins has been thoroughly studied (*8*−*12*). On the other hand, procyanidins, in particular the more polymerized ones, are not readily absorbed in the intestine, remaining in the intestinal lumen for long periods of time (*13*). These two facts make procyanidins potentially good inhibitors of digestive enzymes. In the past, the inhibition of digestive enzymes by phenolic compounds, particularly procyanidins, has been reported (*14*−*16*). Reports of this type are still scarce in the exact mechanism through which polyphenol interaction with enzymes leads to the inhibition of their activity.

In the present paper the interaction between procyanidins and trypsin is studied using fluorescence quenching, dynamic light scattering (DLS), nephelometry, and circular dichroism to understand the enzymatic inhibition previously reported (*14*).

MATERIALS AND METHODS

Reagents. Trypsin (type IX-S from porcine pancreas) and *N*-benzoyl-DL-arginine-*p*-nitroanilide (BApNA) were obtained from Sigma Aldrich. Trypsin was dissolved at room temperature in phosphate buffer (50 mM, pH 7.0). BApNA was dissolved in DMSO (44 $g \cdot L^{-1}$) to prepare a stock solution that was stored at 0 °C. BApNA working solutions (2.5 $g \cdot L^{-1}$) were prepared immediately before use by dilution of the stock solution using distilled water.

Grape Seed Tannin Isolation. Condensed tannins were obtained as described in the literature (*17*). Briefly, *Vitis vinifera* grape seeds were extracted with an ethanol/water/chloroform solution (1:1:2). The chloroform phase containing chlorophylls and lipids was rejected. The resulting hydroalcoholic phase was extracted with ethyl acetate and evaporated,

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yielding a residue comprised of monomeric and oligomeric procyanidins (OPC). These compounds were fractionated through a TSK Toyopearl HW-40(s) gel column (100 mm × 10 mm i.d., with 0.8 mL min^{-1} methanol as eluent) producing four fractions. Fractions 0 and I were obtained after elution with 99.8% (v/v) methanol during 1 and 5 h, respectively (cumulative time); fraction II was obtained after elution with methanol/acetic acid 5% (v/v) during the next 14 h; fraction III was obtained after elution with methanol/10% acetic acid (v/v) during the next 8 h. All fractions were mixed with deionized water and freeze-dried. Fraction 0 was discarded since it was composed by phenolic acids and does not react with trypsin (*14*). The composition in procyanidins of each fraction was determined by direct analysis through ESI-mass spectrometry (Finnigan DECA XP PLUS), as described elsewhere (*18*). Fraction I contains procyanidin trimers and tetramers and their galloyl derivatives (mean MW = 950); fraction II contains procyanidin pentamers and their galloyl derivatives (mean MW = 1512); fraction III contains procyanidin pentamers digalloylated, procyanidin tetramers tetragalloylated, procyanidin hexamers galloylated, procyanidin heptamers, and their galloyl derivatives (mean MW = 2052). The mean molecular weight of the fractions was determined based on the relative abundance of each flavanol present in the fraction.

Fluorescence Quenching Measurements. The quenching effect between trypsin and procyanidin fractions with different molecular weight was assayed using a Perkin-Elmer LS 45 fluorometer. Tryptophan was used as an intrinsic fluorophore (λ_{ex} = 290 nm; λ_{em} = 340 nm). For the quenching assays, the excitation wavelength was set to 290 nm, and the emission spectrum was recorded from 300 to 500 nm.

Fluorescence quenching is described by the Stern−Volmer equation (eq 1), where F_0 and F are the fluorescence intensities in the absence and in the presence of 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 (*19*). Linear Stern−Volmer plots are indicative of a single class of fluorophores in the protein; this also means that only one mechanism (dynamic or static) of quenching occurs. In the case of a dynamic mechanism for fluorescence quenching it is the diffusion-limited collision between the quencher and the fluorophore molecules that allows for energy transfer without radiation (quenching). The static mechanism involves the formation of a complex between the fluorophore and the quencher. In both cases, the bimolecular quenching constant k_q can be calculated by the ratio between K_{SV} and τ_0. The τ_0 for the quenching of tryptophan residues in trypsin is 2.80 ns (*20*).

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

Positive deviations from the Stern−Volmer equation may occur if the extent of quenching is large. In these cases, the Stern−Volmer plot exhibits an upward curvature, concave toward the *y* axis at high [Q]. These positive deviations may be an indication that the fluorophore is being quenched by both mechanisms simultaneously or the presence of a sphere of action (*21*). The sphere of action model assumes the existence of a sphere of volume around a fluorophore within which quenching occurs due to the quencher being adjacent to the fluorophore at the moment of excitation. The modified form of the Stern−Volmer equation that describes this situation is presented in eq 2 and allows the calculation of a quenching constant that is referred to as the apparent static quenching constant (K_{app}). In this equation, V is the volume of the sphere and N is Avogadro's constant (*22*).

$$\frac{F_0}{F} = (1 - K_{app}[Q])\exp([Q]VN/1000) \quad (2)$$

To evaluate the possibility of fluorescence resonance energy transfer (FRET) between the protein and the procyanidins, the absorption spectra of both were analyzed (data not shown): trypsin absorbs light between 200 and 290 nm. At this wavelength the procyanidins do not emit light (maximum at 330 nm). Procyanidin fractions have an absorption maximum at 270 nm, and their spectrum decreases to base levels sharply reaching residual values at 310 nm. The protein emission spectrum starts at 320 nm, and at this λ the polyphenol absorbance is residual. Therefore, it does not seem likely that energy transference between these two molecules may occur.

The experiments were performed in 50 mM phosphate buffer as solvent (pH 7.0). A pH of 7.0 was chosen because it is the one that occurs in the duodenum after neutralization of gastric fluids (*23*). In several microtubes, a volume of procyanidin fraction stock solution in phosphate buffer was mixed with a fixed volume of trypsin stock solution, the microtube was shaken, and the emission spectra were measured in the fluorometer cell. Since the procyanidin fractions possess intrinsic fluorescence at the λ_{ex} (290 nm), their spectrum was measured and subtracted in all fluorescence experiments.

Dynamic Light Scattering Measurements. Both size and total number of aggregates present in solution were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern). In this device the sample solution is illuminated by a 633 nm laser, and the intensity of light scattered at an angle of 173° is measured by an avalanche photodiode. The fluctuations in the intensity of scattered light as particles undergo Brownian motion are measured. These fluctuations are then used to calculate the diffusion coefficients of particles, which are converted into a size distribution. This analysis provides information concerning particle size (obtained by the parameter number) and the amount of scattered light (count rate). Different volumes of procyanidin fraction stock solution were mixed with a fixed volume of trypsin stock solution and allowed to react for 1 h. After this, the microtube was shaken, its content was transferred to a DLS plastic disposable cell, and the measurement was performed. All solutions were thoroughly filtered through 0.2 μm disposable PTFE filters before mixing.

Nephelometry Measurements. In the nephelometry experiments, a Perkin-Elmer LS 45 fluorometer was used as a 90° light scattering photometer. Both excitation and emission wavelengths were the same (400 nm), enabling the determination of light scattered by particles in solution. This wavelength corresponds to the minimum value at which both protein and procyanidins do not absorb the incident light, improving the response of the technique (*24*). The experimental work was conducted in the same manner as for the fluorescence quenching assays.

Enzymatic Activity Assays. Trypsin activity was assayed using *N*-benzoyl-DL-arginine-*p*-nitroanilide (BApNA) as substrate according to the method described in the literature with slight modifications (*14*, *25*). The release of *p*-nitroaniline after tryptic action was detected at 410 nm using a UV−vis microplate reader (Biotek Powerwave XS). The concentration of BApNA used was 360 μM, and that of trypsin was 3 μM since these proved to be appropriate to calculate the initial rate from the graphics of ΔAbs = *f*(time) in the experimental conditions used. The temperature was set to 37 °C. From the plots of relative rate vs procyanidin concentration the inhibitory factor was calculated and used to compare the inhibitory ability of the procyanidin fractions (*14*).

Circular Dichroism (CD). A Jasco J-710 spectropolarimeter running the software Jasco J715CD was used to collect spectra at wavelengths 190−240 nm (far-UV) and 240−300 nm (near-UV). Quartz cuvettes of path lengths 1 mm and 10 mm were used for the far-UV and near-UV regions, respectively. The scanning speed was 20 nm/min, the response was 4 s, and the bandwidth was 1 nm. Volumes of trypsin and procyanidin fractions dissolved in phosphate buffer (5 mM, pH 7.0) were added to achieve a molar ratio of 1:1 at a fixed protein concentration of 10 μM. Baseline corrections were performed by subtracting the spectra of the buffer and phenolic compounds from the sample spectra. The data are reported as mean residue ellipticity, [Θ] in deg·cm^2·$dmol^{-1}$. Each spectrum is the result of three accumulations. The CD spectra were analyzed using the DicroWeb server (*26*) and applying the CONTIN/LL program (*27*) with a reference database, SP175, containing 70 proteins with complex structures including trypsin (*28*).

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 various groups was evaluated by one-way analysis variance (ANOVA) followed by the Tukey test. Differences were considered to be significant when $P < 0.05$. All statistical data were processed using the GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS AND DISCUSSION

A previous work referred that trypsin is considerably inhibited by procyanidins (*14*). The exact process through which procyanidins

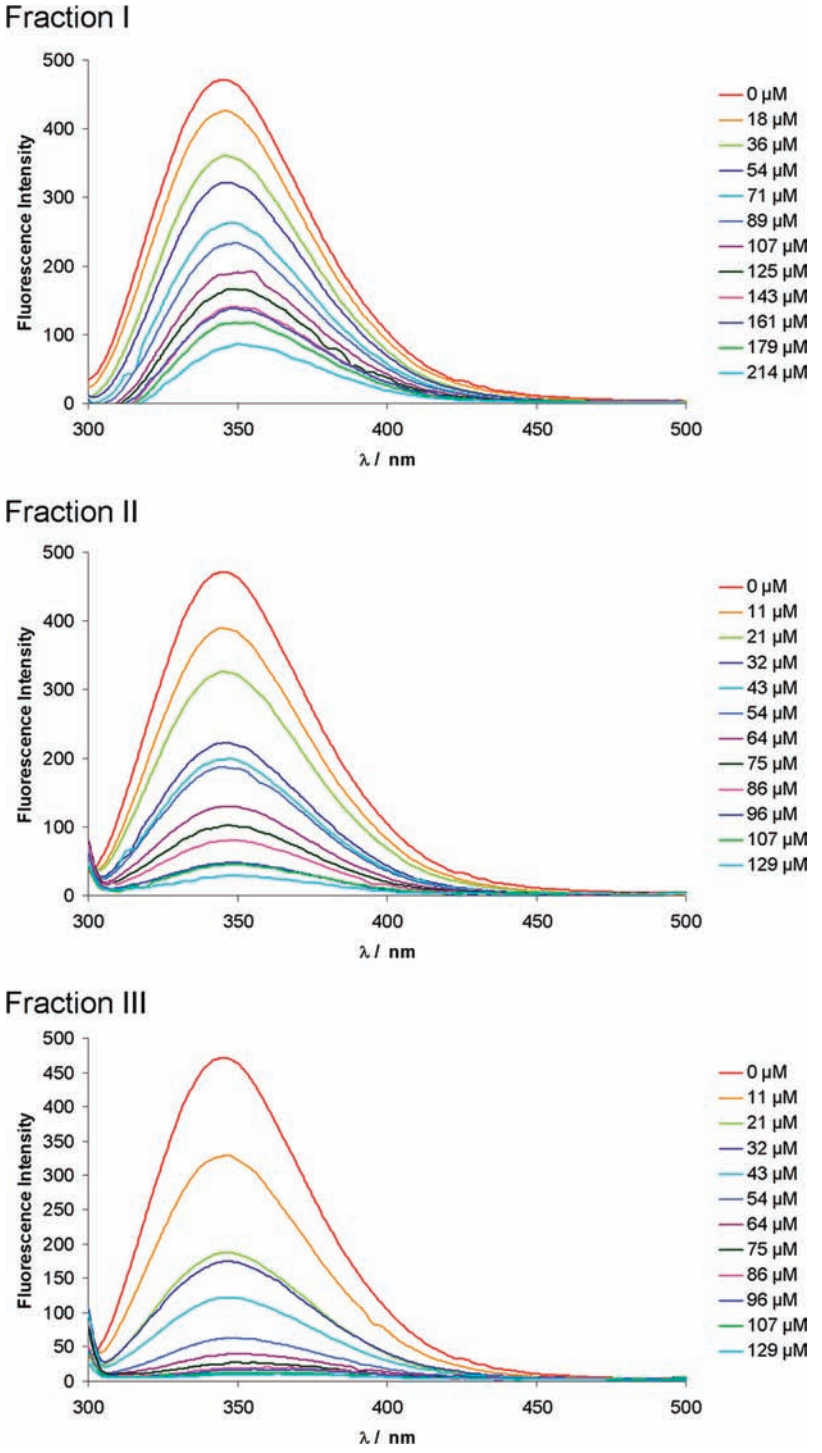

Figure 1. Fluorescence emission spectrum (at λ_{ex} = 290 nm) of trypsin (3 μM) in the presence of increasing concentrations of procyanidin fractions I (mean M_w = 950), II (mean M_w = 1512), and III (mean M_w = 2052) in 50 mM phosphate buffer (pH 7.0). Each curve represents a triplicate assay after correction for polyphenol fluorescence.

exert this inhibitory effect remains unclear. The interactions between trypsin and oligomeric procyanidin fractions were, in the present work, evaluated by the measurement of the intrinsic fluorescence intensity of protein tryptophan residues. The interaction between procyanidin fractions and the digestive protease trypsin was further studied using dynamic light scattering (DLS), nephelometry, and enzyme activity assays.

Fluorescence Quenching. The model protein used in this study was porcine pancreatic trypsin (PPT) that consists of 223 amino acid residues in a single polypeptide of globular shape (*29*) containing four tryptophan residues with intrinsic fluorescence (Trp 51, Trp 141, Trp 215, and Trp 237) (*30*). Among those residues only Trp 51 contributes significantly to the overall fluorescence since all of the others are close to cysteine residues and suffer intramolecular quenching (*29*). 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 (*19*).

The observed λ_{em} for trypsin (λ_{em} = 345 nm) is slightly higher that the λ_{em} for the indole group of tryptophan when isolated

(λ_{em} = 340 nm) (**Figure 1**). This slight shift to longer wavelengths (red shift) indicates that the residue that fluoresces in this protein is located on the surface of the molecule and has contact with solvent molecules (*19*).

The addition of procyanidins to trypsin caused a reduction of trypsin fluorescence intensity by quenching without affecting the emission maximum wavelength (**Figure 1**). This suggests that there was no change in the vicinal 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 could mean that the molecular conformation of the protein was not significantly affected, regardless of the polyphenol mechanism of interaction.

The fluorescence intensity at the trypsin emission maximum was determined from the spectra in **Figure 1**. The plot of F_0/F vs procyanidin concentration, where F_0 and F are the fluorescence intensities before and after the addition of the quencher, is known as a Stern−Volmer plot (**Figure 2**). 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 present case, a linear Stern−Volmer plot was observed for procyanidin fraction I. From the Stern−Volmer plots, a constant known as the Stern−Volmer constant may be calculated using eq 1 (**Table 1**).

This constant is a measure of the ability of the tested polyphenols to interact with the protein in solution, reducing the fluorescence of the amino acid residue that is fluorescing (in this case tryptophan) and is determined as the slope of the $F_0/F = f(|\text{quencher}|)$ plot. Procyanidin fraction I has a high value for the Stern−Volmer constant, which is consistent with a static quenching mechanism.

For the two more polymerized procyanidin fractions the Stern−Volmer plot exhibited an upward curvature, concave toward the *y* axis, at high procyanidin concentration. This positive deviation may be an indication that the protein is suffering both static (complex forming) and dynamic (collision induced) quenching, or it could mean the existence of a "sphere of action" (*21*). In this case the Stern−Volmer plot may be linearized according to eq 2 that corresponds to a sphere of action model involving a static mechanism of quenching, by representing $\ln(F_0/F)$ vs |quencher| (**Figure 3**). The slope of this plot corresponds to the apparent static quenching constant (K_{app}) (**Table 1**). From the analysis of **Figure 3** and **Table 1**, it is clear that the ability of procyanidins to quench the fluorescence of trypsin increases with their M_w.

Even though fraction I presented a linear Stern−Volmer plot, a linearization using the Stern−Volmer modification described in the Materials and Methods section was performed for clarity of comparison. It is important to investigate if any specific interaction occurs between trypsin (fluorophore) and procyanidins (quencher). If this interaction takes place, a complex that does not display fluorescence after returning from the excited state may be formed between the two compounds. To verify this, the apparent bimolecular quenching constant was calculated, dividing K_{app} by the lifetime of the fluorophore in the absence of quencher (τ_0) (**Table 1**). The maximum value possible for diffusion-limited quenching (dynamic mechanism) in water is 10^{10} M^{-1} s^{-1}. When the value of the bimolecular quenching constant is higher, it is indicative of the formation of a complex between protein and quencher, corresponding to a static mechanism (*31*). The results obtained with these procyanidin fractions indicate that the association of procyanidins with trypsin involves the formation of a stable interaction between the two involving quenching by either a static or a sphere of action model that accounts for the high values of the quenching constants. These are in very close relation to the activity inhibition observed for trypsin interaction with procyanidins (**Table 1**). This indicates that the same association of procyanidin molecules to trypsin that quenches fluorescence also reduces its enzymatic activity.

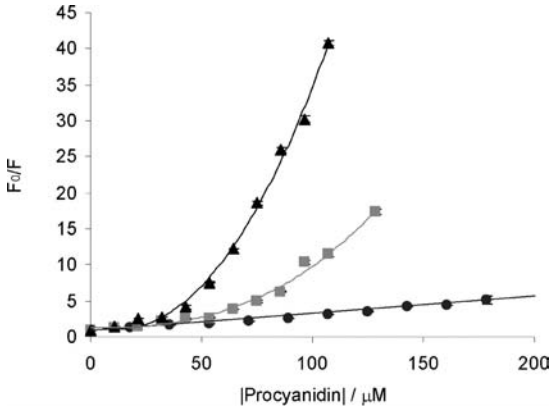

Figure 2. Stern−Volmer plot describing tryptophan quenching of trypsin (3 μM) at pH 7.0 by increasing concentrations of oligomeric procyanidin fractions (●, FI; shaded box, FII; ▲, FIII). The fluorescence emission intensity was recorded at λ_{ex} 290 nm, and the λ_{em} maximum occurred at 345 nm. Solid lines correspond to the logaritmic regression of the Stern−Volmer equation (eq 1).

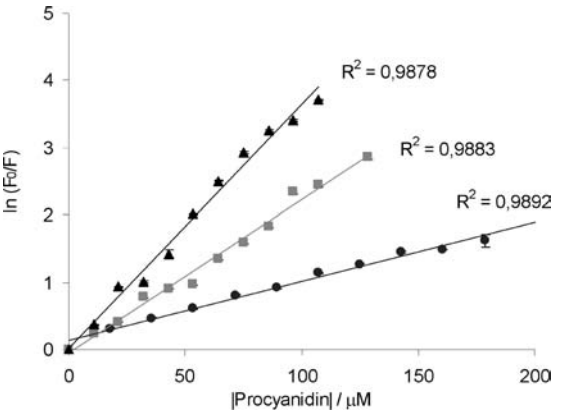

Figure 3. Modified Stern−Volmer plot describing tryptophan quenching of trypsin (3 μM) at pH 7.0 by increasing concentrations of oligomeric procyanidin fractions (●, FI; shaded box, FII; ▲, FIII). Solid lines correspond to the linear regression of the modified Stern−Volmer equation (eq2).

Table 1. Stern−Volmer (K_{SV}) and Bimolecular (k_q) Quenching Constants, Apparent Static Quenching Constant (K_{app}), Apparent Bimolecular (k_q^{app}) Quenching Constant, and IC_{50} for the Interaction of Trypsin and Procyanidin Fractions with Increasing Degree of Polymerization[a]

	K_{SV} (M^{-1})	$k_q \times 10^{-10}$ (M^{-1} s^{-1})[b]	K_{app} (M^{-1})	$k_q^{app} \times 10^{-10}$ (M^{-1} s^{-1})	IC_{50} (μM)
fraction I	26890 ± 553	960 ± 20[b]	8858 ± 142 a		2883 ± 103 a
fraction II	nd	nd	22730 ± 119 b	812 ± 4 a	182 ± 8 b
fraction III	nd	nd	33280 ± 108 b	1188 ± 4 b	118 ± 5 c

[a] K_{SV} was only calculated for fraction I since other fractions have nonlinear Stern−Volmer plots. Values with different letters are significantly different ($P < 0.05$). [b] Calculated from K_{SV} (if calculated from K_{app}, the k_q would be $310 \times 10^{10} \pm 5 \times 10^{10}$).

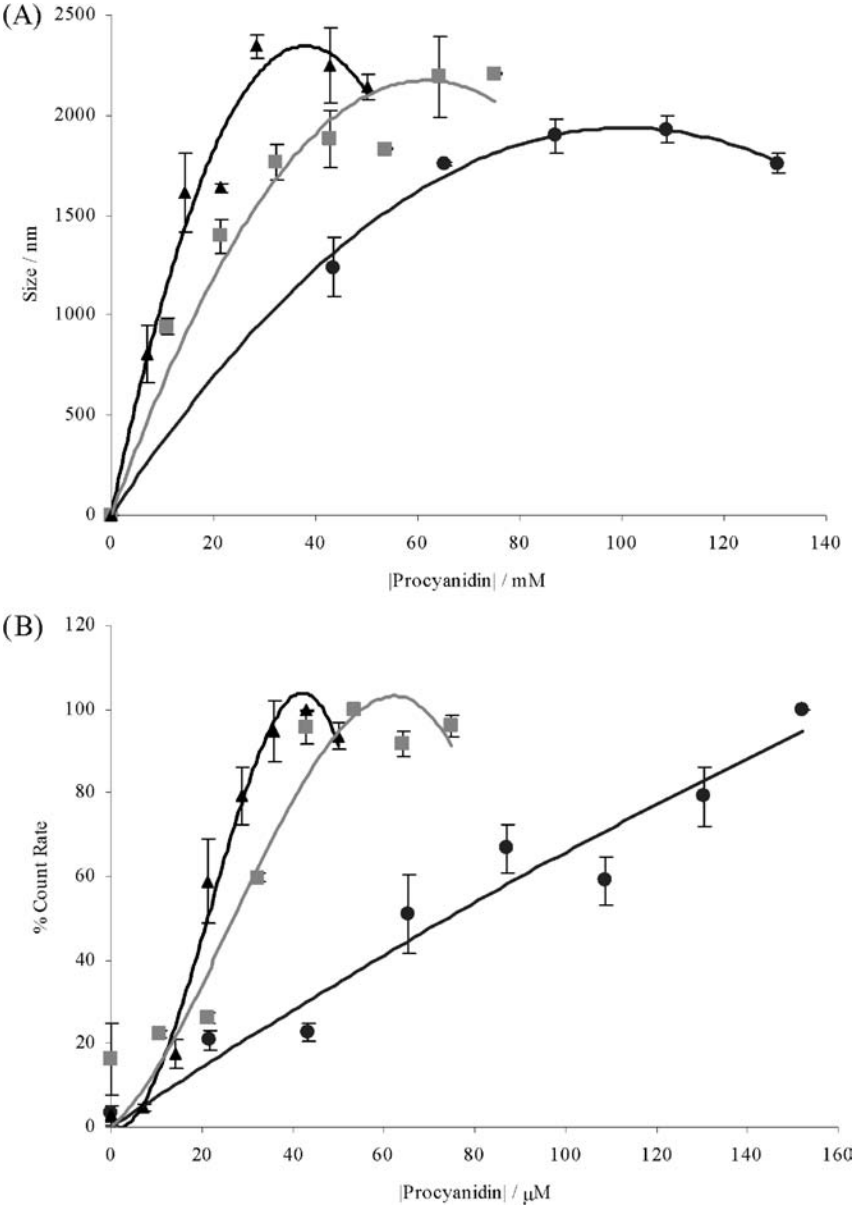

Figure 4. Changes in trypsin (3 μM)/procyanidin aggregate size (**A**) and aggregation intensity by count rate (**B**) by increasing concentrations of oligomeric procyanidin fractions (●, FI; shaded box, FII; ▲, FIII) at pH 7.0. Solid lines correspond to polynomial fitting of the experimental data.

Differences in the structural modifications induced in the enzyme upon attachment of the polyphenol may be responsible for the slight difference in enzymatic activity loss.

Light Scattering Studies. The interaction between procyanidins and trypsin leads to the formation of aggregates that may be measured directly in solution by light scattering techniques.

The average size of trypsin/procyanidin aggregates was measured using dynamic light scattering (DLS). This technique allows the determination of the diffusion coefficients of particles, which are converted into a size distribution. This result may be calculated using the particles that scatter more light (intensity), those with the greater volume (volume), or the most abundant particles (number). In the present case, the number distribution was used since the most abundant particles in solution are expected to be the aggregates between procyanidins and protein.

The representation of size vs procyanidin concentration shows an increase in aggregate size with increasing concentration of procyanidin fractions (**Figure 4A**). The maximum particle size achieved is very close for the three fractions of procyanidins. The molecular weight of procyanidins does not seem to affect size of particles. The more polymerized procyanidin fractions reached the maximum size of procyanidin/trypsin aggregates for lower procyanidin concentrations (120 μM for FI, 60 μM for FII, and 40 μM for FIII). After this maximum value, corresponding to the stoichiometry of the aggregates, the size decreases slightly. This decrease could be explained by a model in which excessive procyanidin concentration causes a resolubilization of aggregates resulting from a reorganization of their stoichiometry (*32*).

Figure 4B shows the amount of light scattered by the particles in solution whatever their size. The representation of count rate vs procyanidin concentration is similar to the analysis of size, in particular for fractions II and III. An increase in the count rate is observed with increasing procyanidin concentrations. For fractions II and III a maximum of light scattering occurs at the same concentration as for maximum size. However, for low molecular weight procyanidins (fraction I) the increase in count rate is linear throughout the concentration range studied (0−160 μM). This increase in intensity, even when aggregate size does not change or decreases slightly, may result from a higher number of these procyanidin/trypsin aggregates.

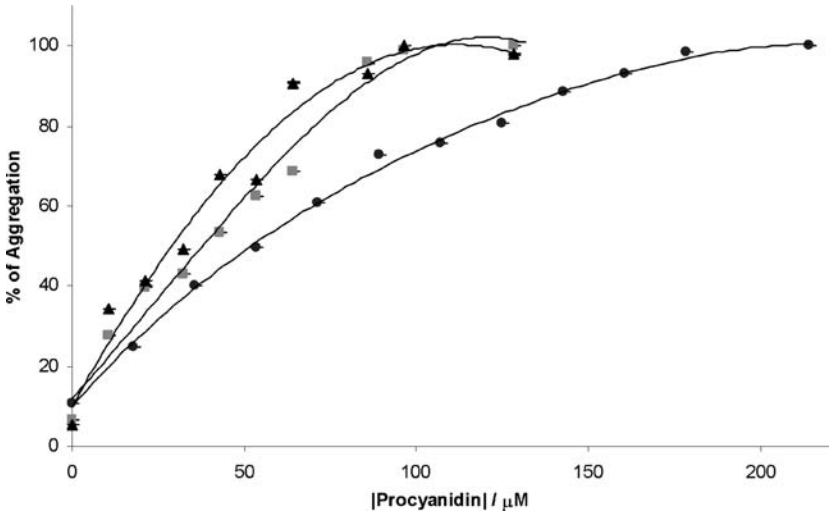

Figure 5. Changes in trypsin (3 μM)/procyanidin aggregation intensity by nephelometry with increasing concentrations of oligomeric procyanidin fractions (●, FI; shaded box, FII; ▲, FIII) at pH 7.0. Solid lines correspond to polynomial fitting of the experimental data.

To confirm these results, the light scattered by aggregates in solution was also measured by nephelometry (**Figure 5**). This value is directly related to the measure of count rate using DLS.

Again, there is an increase in protein aggregating ability with increasing molecular weight of procyanidins. Indeed, the more polymerized procyanidin fractions are able to induce aggregation of trypsin at lower concentration. A maximum of turbidity was achieved near the concentration of procyanidins found with DLS and corresponding to the stoichiometry of the complexes. After this maximum the turbidity values remain more or less constant or decrease slightly. Again, fraction I was shown to have a slightly different behavior, and the turbidity was shown to increase regularly and slowly with the increase in procyanidin concentration indicating that a steady state was achieved.

Circular Dichroism Studies. Circular dichroism has been used in the past to evaluate alterations in protein structure occurring after interaction with phenolic compounds (*11*, *33*, *34*). CD spectra of trypsin in the absence and presence of oligomeric procyanidin fractions (1:1 molar ratio) were recorded in phosphate buffer (5 mM) at pH 7.0 and room temperature. The CD contribution of the oligomeric procyanidin fractions was removed by independently recording the procyanidin spectra in solution, as reported for other flavan-3-ols (*34*).

The far-UV CD spectrum of trypsin exhibits two negative bands in the UV region at 208 and 220 nm (**Figure 6A**) which are compatible with a normally folded active enzyme (*35*). Trypsin belongs to the serine protease family, and its structure is similar to α-chymotrypsin. X-ray structure analysis has shown that α-chymotrypsin consists of distorted antiparallel pleated β-sheets, forming short irregular strands. In general, the β-sheet shows a CD band at 210−220 nm. However, in that case, these irregular strands caused the negative CD band to shift from the ideal β-sheet position toward the 200 nm region (*35*). This could explain why the maximum band of trypsin is located at 208 nm even though this enzyme has a structure where β-sheets are predominant. The interaction of trypsin with the different procyanidin fraction induced some degree of structural modification in the enzyme as shown by the variation of the far-UV CD spectra upon interaction (**Figure 6A**).

CD spectra were also analyzed using the CONTIN program to estimate the secondary structure adopted by the protein in the absence and in the presence of the different procyanidin fractions (**Table 2**). The secondary structure estimation obtained for trypsin

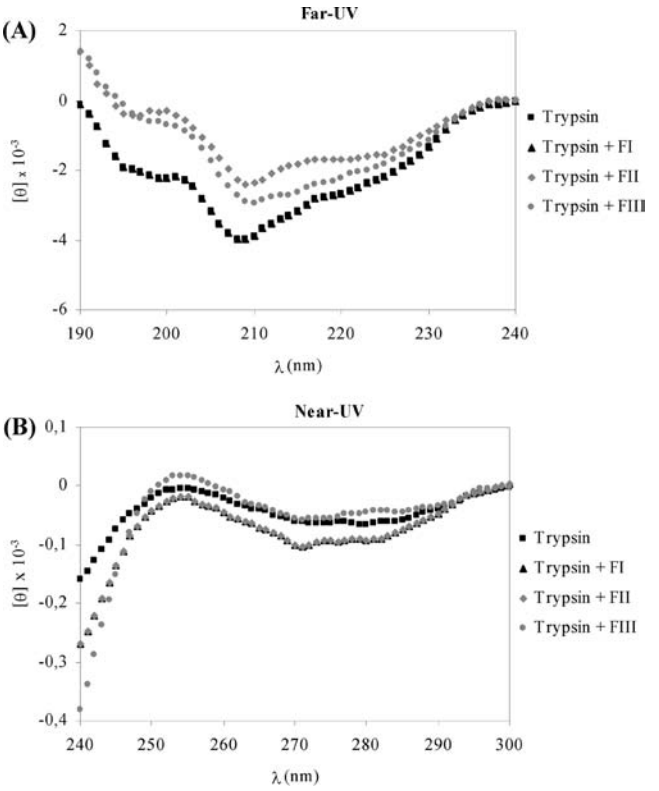

Figure 6. CD spectra of trypsin (10 μM) and the 1:1 trypsin/procyanidin complex for the oligomeric procyanidin fractions. (**A**) 190−240 nm. (**B**) 240−300 nm.

alone presents a good relation to the literature data, indicating a large predominance of β-sheet structure (*35*). Upon addition of procyanidins very small changes in the secondary structure of the enzyme were detected. These consist of a reduction of the unordered portion of the protein structure accompanied by an increase in the content of β-sheet. This very small variation of secondary structure also explains why fluorescence quenching occurs without a significant modification of the emission maximum of the protein (**Figure 1**).

The influence of procyanidin binding on tertiary structure was also studied by near-UV circular dichroism (**Figure 6B**).

Table 2. Percentages of Secondary Structural Elements in Trypsin upon Interaction with Procyanidin Fractions with Increasing Degree of Polymerization[a]

	α-helix	β-sheet	turn	unordered
trypsin	7.8	39.8	12.7	39.8
trypsin + fraction I	7.7	40.0	12.7	39.5
trypsin + fraction II	7.7	40.2	12.7	39.4
trypsin + fraction III	7.7	40.2	12.7	39.4

[a] Data calculated using the CONTIN program implementing the Provencher and Glöckner curve-analyzing algorithm (*27*).

Slight changes in the near-UV CD spectra were observed for all of the tested procyanidin fractions. This is likely the result of conformational restriction of the aromatic residue side chains. CD spectra were significantly different throughout the 240−290 nm range where tryptophan, tyrosine, and phenylalanine side chains as well as the six disulfide bonds present in trypsin are optically active. This suggests that, upon binding, procyanidins are close to these groups. This does not mean, however, that they are directly involved in the binding process.

Concluding, the interaction between procyanidins and trypsin increases with increasing degree of polymerization of procyanidins. This interaction seems to occur by a specific interaction between protein and polyphenol following the static mechanism (fraction I) or the sphere of action mechanism (fractions II and III) depending on the molecular weight of procyanidins. This interaction is also accompanied by the formation of insoluble aggregates detected by dynamic light scattering and nephelometry and closely parallels the inhibition of trypsin hydrolytic activity observed (**Table 1**). The binding of procyanidins to trypsin is also detectable by CD and has a small effect in both secondary and tertiary structure for this protein.

In the digestive system this inhibition may contribute to a reduction in the hydrolysis of protein, yielding a reduced weight gain in animals. On the other hand, it may contribute to longer digestion times that lead to a sensation of fullness that may reduce overall food intake, contributing to control obesity.

This result may be relevant not only to study the antinutritional properties of procyanidin-containing foods but also to study new protease inhibitors for therapeutic purposes and also to assess the health benefits of procyanidin-rich (or enriched) foods and beverages.

LITERATURE CITED

(1) Leung, D.; Abbenante, G.; Fairlie, D. P. Protease inhibitors: Current status and future prospects. *J. Med. Chem.* **2000**, *43*, 305−341.

(2) Weiss, F. U.; Halangk, W.; Lerch, M. M. New advances in pancreatic cell physiology and pathophysiology. *Best Pract. Res., Clin. Gastroenterol.* **2008**, *22*, 3−15.

(3) Manach, C.; Scalbert, A.; Morand, C.; Remesy, C.; Jimenez, L. Polyphenols: Food sources and bioavailability. *Am. J. Clin. Nutr.* **2004**, *79*, 727−747.

(4) Gee, J. M.; Johnson, I. T. Polyphenolic compounds: Interactions with the gut and implications for human health. *Curr. Med. Chem.* **2001**, *8*, 1245−1255.

(5) Mennen, L. I.; Walker, R.; Bennetau-Pelissero, C.; Scalbert, A. Risks and safety of polyphenol consumption. *Am. J. Clin. Nutr.* **2005**, *81*, 326S−329S.

(6) McDougall, G. J.; Stewart, D. The inhibitory effects of berry polyphenols on digestive enzymes. *Biofactors* **2005**, *23*, 189−195.

(7) McDougall, G. J.; Kulkarni, N. N.; Stewart, D. Current developments on the inhibitory effects of berry polyphenols on digestive enzymes. *Biofactors* **2008**, *34*, 73−80.

(8) Baxter, N. J.; Lilley, T. H.; Haslam, E.; Williamson, M. P. Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. *Biochemistry* **1997**, *36*, 5566−5577.

(9) Charlton, A. J.; Baxter, N. J.; Khan, M. L.; Moir, A. J.; Haslam, E.; Davies, A. P.; Williamson, M. P. Polyphenol/peptide binding and precipitation. *J. Agric. Food Chem.* **2002**, *50*, 1593−1601.

(10) de Freitas, V.; Mateus, N. Structural features of procyanidin interactions with salivary proteins. *J. Agric. Food Chem.* **2001**, *49*, 940−945.

(11) Jobstl, E.; O'Connell, J.; Fairclough, J. P. A.; Williamson, M. P. Molecular model for astringency produced by polyphenol/protein interactions. *Biomacromolecules* **2004**, *5*, 942−949.

(12) Soares, S. I.; Gonçalves, R. M.; Fernandes, I.; Mateus, N.; de Freitas, V. Mechanistic approach by which polysaccharides inhibit α-amylase/procyanidin aggregation. *J. Agric. Food Chem.* **2009**, *57*, 4352.

(13) Nakamura, Y.; Tonogai, Y. Metabolism of grape seed polyphenol in the rat. *J. Agric. Food Chem.* **2003**, *51*, 7215−7225.

(14) Gonçalves, R.; Soares, S.; Mateus, N.; de Freitas, V. Inhibition of trypsin by condensed tannins and wine. *J. Agric. Food Chem.* **2007**, *55*, 7596−7601.

(15) Matsui, T.; Ueda, T.; Oki, T.; Sugita, K.; Terahara, N.; Matsumoto, K. α-glucosidase inhibitory action of natural acylated anthocyanins. 2. α-glucosidase inhibition by isolated acylated anthocyanins. *J. Agric. Food Chem.* **2001**, *49*, 1952−1956.

(16) Rohn, S.; Rawel, H. M.; Kroll, J. Inhibitory effects of plant phenols on the activity of selected enzymes. *J. Agric. Food Chem.* **2002**, *50*, 3566−3571.

(17) de Freitas, V.; Glories, Y.; Bourgeois, G.; Vitry, C. Characterisation of oligomeric and polymeric procyanidins from grape seeds by liquid secondary ion mass spectrometry. *Phytochemistry* **1998**, *49*, 1435−1441.

(18) González-Manzano, S.; Mateus, N.; de Freitas, V.; Santos-Buelga, C. Influence of the degree of polymerisation in the ability of catechins to act as anthocyanin copigments. *Eur. Food Res. Technol.* **2008**, *227*, 83−92.

(19) Lakowicz, J. R. *Principles of fluorescence spectroscopy*, 2nd ed.; Kluwer Academic/Plenum Publishers: New York, 1999.

(20) Lakowicz, J. R.; Weber, G. Quenching of fluorescence by oxygen. Probe for structural fluctuations in macromolecules. *Biochemistry* **1973**, *12*, 4161−4170.

(21) Papadopoulou, A.; Green, R. J.; Frazier, R. A. Interaction of flavonoids with bovine serum albumin: A fluorescence quenching study. *J. Agric. Food Chem.* **2005**, *53*, 158−163.

(22) Lima, S. A. C.; Cordeiro-da-Silva, A.; de Castro, B.; Gameiro, P. Sensitivity of p-glycoprotein tryptophan residues to benzodiazepines and ATP interaction. *Biophys. Chem.* **2007**, *125*, 143−150.

(23) Swarbrick, J.; Boylan, J., *Encyclopedia of pharmaceutical technology*; Marcel Dekker: New York, 1998; Vol. 5, pp 235−263.

(24) Carvalho, E.; Mateus, N.; Plet, B.; Pianet, I.; Dufourc, E.; de Freitas, V. Influence of wine pectic polysaccharides on the interactions between condensed tannins and salivary proteins. *J. Agric. Food Chem.* **2006**, *54*, 8936−8944.

(25) Erlanger, B. F.; Kokowsky, N.; Cohen, W. The preparation and properties of two new chromogenic substrates of trypsin. *Arch. Biochem. Biophys.* **1961**, *95*, 271−278.

(26) Whitmore, L.; Wallace, B. A. Protein secondary structure analyses from circular dichroism spectroscopy: Methods and reference databases. *Biopolymers* **2008**, *89*, 392−400.

(27) Provencher, S. W.; Gloeckner, J. Estimation of globular protein secondary structure from circular dichroism. *Biochemistry* **1981**, *20*, 33−37.

(28) Lees, J. G.; Miles, A. J.; Wien, F.; Wallace, B. A. A reference database for circular dichroism spectroscopy covering fold and secondary structure space. *Bioinformatics* **2006**, *22*, 1955−1962.

(29) Koutsopoulos, S.; Patzsch, K.; Bosker, W. T. E.; Norde, W. Adsorption of trypsin on hydrophilic and hydrophobic surfaces. *Langmuir* **2007**, *23*, 2000−2006.

(30) Zhang, H.-M.; Wang, Y.-Q.; Zhou, Q.-H. Investigation of the interactions of quercetin and morin with trypsin. *Luminescence* **2009**, *24*, 355−362.

(31) Rasoulzadeh, F.; Jabary, H. N.; Naseri, A.; Rashidi, M.-R. Fluorescence quenching study of quercetin interaction with bovine milk xanthine oxidase. *Spectrochim. Acta, Part A* **2009**, *72*, 190−193.

(32) aslam, E.; Lilley, T. H. Natural astringency in foodstuffs-a molecular interpretation. *Crit. Rev. Food Sci. Nutr.* **1988**, *27*, 1−40.
(33) Pascal, C.; Poncet-Legrand, C.; Imberty, A.; Gautier, C.; Sarni-Manchado, P.; Cheynier, V.; Vernhet, A. Interactions between a nonglycosylated human proline-rich protein and flavan-3-ols are affected by protein concentration and polyphenol/protein ratio. *J. Agric. Food Chem.* **2007**, *55*, 4895−4901.
(34) Simon, C.; Barathieu, K.; Laguerre, M.; Schmitter, J.-M.; Fouquet, E.; Pianet, I.; Dufourc, E. J. Three-dimensional structure and dynamics of wine tannin-saliva protein complexes. A multitechnique approach. *Biochemistry* **2003**, *42*, 10385−10395.
(35) Simon, L. M.; Kotormán, M.; Garab, G.; Laczkó, I. Structure and activity of α-chymotrypsin and trypsin in aqueous organic media. *Biochem. Biophys. Res. Commun.* **2001**, *280*, 1367−1371.

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