Study of the Interaction of Pancreatic Lipase with Procyanidins by Optical and Enzymatic Methods

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The interactions between porcine pancreatic lipase (PL) and grape seed procyanidins were studied by an enzymatic assay, fluorescence quenching, nephelometry, and dynamic light scattering (DLS). An inhibitory effect of grape seed procyanidins on lipase hydrolytic activity was found. Both the inhibition of lipase activity by procyanidins and the respective quenching of intrinsic protein fluorescence increased with the average degree of polymerization of the tested procyanidins. The association between procyanidins and enzyme involves a specific interaction as inferred from the fluorescence assays despite not changing significantly the tertiary structure of the protein. For all tested procyanidins it was shown, both by DLS and by nephelometry, that an increase in aggregation occurs up to a stoichiometric maximum after which further procyanidin addition causes a decrease in aggregation of aggregates. The maximum size of aggregates was shown to be closely related to the maximum overall aggregation. It was also shown that the inhibition of enzyme activity is to a large extent independent of the formation of aggregates.

KEYWORDS: Pancreatic lipase; procyanidin; fluorescence quenching; dynamic light scattering; anti-nutritional effect

INTRODUCTION

Excessive calorie intake is associated with the occurrence of obesity, a risk factor of cardiovascular disease, diabetes, cancer, and metabolic syndrome (*1*). Obesity and overweight are also the most prevailing nutritional diseases in developed nations (*2*). An effective way to prevent obesity is to inhibit fat absorption from the intestine or increase metabolic rate and fat oxidation (*3*). Triacylglycerols are a calorie-rich energy source whose suppression of absorption is directly associated with the prevention of obesity and obesity-related diseases (*4*). One of the most important enzymes involved in lipid metabolism is pancreatic lipase (PL, E.C. 3.1.1.3) (*5*). This enzyme hydrolyzes triacylglycerols into glycerol and fatty acids, allowing their absorption in the small intestine (*6*).

Bearing this, the suppression of triacylglycerol absorption by inhibiting lipase is a major approach to avert obesity. In the past years this has led to the usage of Orlistat as an agent for obesity managment. Orlistat inhibits triacylglycerol absorption by the inhibition of pancreatic lipase, and its long-term administration results in weight loss, suggesting its efficacy for the treatment of obesity (*7*). It is however associated with side effects such as decreased vitamin D absorption and myopathy (*8*, *9*), indicating that alternatives to its usage should be researched.

There are several works in the literature concerning the inhibition of digestive lipases by epigallocatechin and similar compounds mostly because of the obesity control effect attributed to tea (*3*, *10*, *11*). However, as far as procyanidins are

concerned the evidence is still scarce (*12–14*). Procyanidins (PC) are a class of polyphenols that enter the human diet mainly through fruits and vegetable-based beverages like juices, beer, and wine (*15*). They are polymeric flavan-3-ols whose elementary units are linked by C–C and occasionally C–O–C bonds yielding large ramified structures (*16*). Their capacity to interact with enzymes causing their inhibition is considered as one mechanism through which polyphenols exert their effect and might be one of the explanations to the beneficial effects of procyanidins in health (*16*).

The aim of the present work was to assess the effects of grape seed procyanidins on the lipid metabolizing enzyme pancreatic lipase. The effect of procyanidins on enzymatic activity was studied, aiming to relate this effect with the degree of polymerization of procyanidins.

MATERIALS AND METHODS

Materials. Lipase (PL), type II from porcine pancreas, and *p*-nitrophenyl caprylate (pNPC) were purchased from Sigma-Aldrich Chemie GMbH (Steinheim, Germany). Lipase (19 μM) was dissolved in phosphate buffer (50 mM, pH 7.0), aliquoted, and frozen. Prior to use PL was centrifuged (7000*g*; 2 min). pNPC was dissolved in Triton X-100/phosphate buffer (1:40) and stored at 4 °C.

Grape Seed Tannins. 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 hydroalcoholic phase was further extracted with ethyl acetate, yielding a mixture of monomeric and oligomeric procyanidins (OPC). These were fractionated through a TSK Toyopearl HW-40(s) gel column producing three fractions (*18*). The composition in procyanidins of each fraction was determined by direct analysis through ESI-MS (Finnigan DECA XP PLUS) and is presented in

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Table 1. ESI/MS Analysis of Oligomeric Procyanidin Fractions[a]

	mol wt (m/z) [M − H]⁻	identified flavonoids	rel abundance (%)	mean mol wt
fraction I	289	catechin	5	777
	441	catechin gallate	3	
	577	PC dimer	12	
	729	PC dimer gallate	39	
	865	PC trimer	15	
	1017	PC trimer gallate	16	
	1154	PC tetramer	10	
fraction II	577	PC dimer	10	936
	729	PC dimer gallate	23	
	865	PC trimer	28	
	1017	PC trimer gallate	12	
	1153	PC tetramer	12	
	1305	PC tetramer gallate	8	
	1594	PC pentamer gallate	6	
fraction III	729	PC dimer gallate	7	1581
	865	PC trimer	4	
	1017	PC trimer gallate	4	
	1153	PC tetramer	10	
	1305	PC tetramer gallate	9	
	1422	PC pentamer	6	
	1594	PC pentamer gallate	6	
	1745	PC pentamer digallate	21	
	1761	PC tetramer tetragallate	7	
	2017	PC heptamer	7	
	2169	PC heptamer gallate	19	

[a] The mean molecular weight of fractions was determined based in the relative abundance of the quasi-molecular ion peak [M − H]⁻ of each flavanol in the fraction.

Table 1, where the mean molecular weight of the fractions was determined based on the relative abundance of the quasi-molecular ion peak of each flavanol present in the fraction (*19*).

Enzymatic Activity Assays. Lipase activity was evaluated by the hydrolysis of *p*-nitrophenyl caprylate (pNPC) spectrophotometrically recorded at 405 nm and at pH 7.0 in a thermostated microplate reader at 37 °C according to previous work (*20*). The concentrations of pNPC and PL were 250 and 2.5 μM, respectively, as determined in preliminary assays. In each well of a 96-well microplate phosphate buffer, pNPC and procyanidin fraction were added. After an automatic shaking step to ensure homogenization the enzyme was added, and the measurement process began. For procyanidin fractions I and II the concentrations ranged from 0 to 200 μM and for fraction III from 0 to 150 μM. IC$_{50}$ were determined through simple linear regression of the plots of Abs = *f*-([concentration]). The experiments were performed in phosphate buffer (50 mM, pH 7.0) to simulate the conditions that occur in the duodenum after neutralization of gastric fluids (*21*). An experiment in which procyanidins were incubated with PL or pNPC separately was conducted to determine if aggregation influenced the measurement at 405 nm corresponding to the enzymatic activity. No significant influence on variability (less than 5%) was observed (data not shown)

Fluorescence Quenching. The quenching effect between lipase and procyanidin fractions with different molecular weight was assayed using a Perkin-Elmer LS 45 fluorometer. The aminoacid tryptophan, which is present in PL, was used as an intrinsic fluorophore (λ_{ex} = 290 nm; λ_{em} 340 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 (*22*).

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

Linear Stern−Volmer plots are indicative that a single class of fluorophores exists in the protein; this also means that only one mechanism (dynamic or static) of quenching occurs (*22*). 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 actual τ_0 for tryptophan quenching in PL was not found in the literature. Since PL and its human homologue share 94% sequence identity and have all of the tryptophan residues conserved, the τ_0 is considered in this work to be equal to that of human pancreatic lipase (1.59 ns) (*23*). 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 (*24*). This last 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 (*25*).

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

During the titration experiments the residual fluorescence intensity of PL was analyzed as a function of procyanidin concentration using the equation previously described for a multisite binding model (*26*) (eq 3), where ΔI_F is the fluorescence intensity variation, I_{Fmax} is the difference between the fluorescence of the protein alone and saturated with tannins, K_d is the dissociation constant expressed in molar, $[T]_i$ is the total concentration of polyphenol able to fix the peptide (in molar), $[P]_i$ is the total concentration of peptide (in molar), and n is the number of polyphenol binding sites. In the experimental conditions used it is assumed that we are below the critical micellar concentration (CMC) for the procyanidins used, and therefore no corrections of $[T]_i$ were in order. This assumption is based on the CMC values determined for trimeric procyanidins by NMR (15−27 mM) (*27*). K_d, n, and I_{max} were calculated for both experiments using a least-squares fitting routine within the software program Microsoft EXCEL.

$$A_{obs} = \frac{A_{max}}{2}\left\{\left(1 - \frac{K_d}{n[P]_i} - \frac{[T]_i}{n[P]_i}\right) - \left(\left[1 - \frac{K_d}{n[P]_i} - \frac{[T]_i}{n[P]_i}\right]^2 - \frac{4[T]_i}{n[P]_i}\right)^{1/2}\right\} \qquad (3)$$

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): PL presents an absorption spectrum at 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 reaching residual values close to 310 nm. The protein emission spectrum starts at 320 nm, and at this λ the polyphenol absorbance is residual. FRET seems highly unlikely at the experimental conditions used.

In several microtubes, a volume of procyanidin fraction stock solutions (FI and FII, 250 μM; FIII, 125 μM) in phosphate buffer (50 mM, pH 7.0) was mixed with PL (2.5 μM) and allowed to react for 1 h. After this, the microtube was shaken, its content was transferred to a fluorometer cell, and the emission spectrum was measured. Since the procyanidin fractions may possess intrinsic fluorescence at the λ_{ex} (290 nm), their spectrum was measured and subtracted in all fluorescence experiments.

Dynamic Light Scattering. The size of aggregates present in solution and the total number of aggregates were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern). DLS measures fluctuations in the intensity of scattered light (a 633 nm laser) when particles undergo Brownian motion. The analysis of these fluctuations enables the determination of the diffusion coefficients of particles that are convertible into a size distribution. This analysis provides information concerning particle size (obtained by the parameter number) and the amount of scattered light (count rate). Procyanidin fraction stock solutions (identical to the fluorescence assays) were mixed with PL (2.5 μM) and allowed to react for 1 h. After this incubation period the samples were

Table 2. IC_{50}, Stern−Volmer (K_{SV}), and Bimolecular (k_q) Quenching Constants, Apparent Static Quenching Constant (K_{app}), and Apparent Bimolecular (k_q^{app}) Quenching Constants for the Interaction of Pancreatic Lipase and Three Procyanidin Fractions with Increasing Molecular Weight[a]

	mean M_w	IC_{50} (μM)	K_{SV} (M^{-1})	$k_q \times 10^{-11}$ ($M^{-1} s^{-1}$)	K_{app} (M^{-1})	$k_q^{app} \times 10^{-11}$ ($M^{-1} s^{-1}$)
fraction I	777	1757 ± 336 a	28549 ± 1012 a	180 ± 6 a	12135 ± 208 a	76 ± 1 a
fraction II	936	313 ± 42 b	48295 ± 393 b	304 ± 2 b	17080 ± 51 b	107 ± 0.3 b
fraction III	1581	141 ± 5 b	75546 ± 426 c	475 ± 3 c	30291 ± 173 c[b]	191 ± 1 c[b]

[a] Values with different superscript letters are significantly different ($P < 0.05$). [b] Value calculated only for comparison purposes since the Stern−Volmer plot was linear.

homogenized by shaking and measured. All solutions were thoroughly filtered through 0.2 μm disposable PTFE filters before mixing.

Nephelometry. The nephelometry experiments were similar to the fluorescence quenching assays except for the detection method. For nephelometry measurements the same Perkin-Elmer LS 45 fluorometer was used as a 90° light scattering photometer after 1 h of reaction time (*28*). Both the excitation and the emission wavelengths were set at 400 nm, at which neither PL nor procyanidins absorb the incident light (*28*). A relative aggregation value (percent) was calculated for each experiment as the ratio between the scatter intensity of the measured sample and that of the most turbid sample.

Statistical Analysis. Statistic data were processed using GraphPad Prism (San Diego, CA). Values are 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.

RESULTS AND DISCUSSION

Enzymatic Activity Assays. The inhibition of pancreatic lipase (PL) activity by procyanidin fractions with increasing degrees of polymerization was studied using an enzymatic assay based on the hydrolysis of *p*-nitrophenyl caprylate (pNPC).

The release of *p*-nitroaniline after hydrolysis of this substrate was followed over time at 405 nm, and the initial portion of the hydrolysis curves was used to calculate the IC_{50} for the different fractions (**Table 2**). A relationship between the inhibition of lipase activity and the increase in average degree of polymerization was observed. This may be explained as being the result of a more extensive interaction of procyanidins with the enzyme since it is accepted that more polymerized procyanidins have a stronger affinity for proteins. The inhibition appears to increase exponentially with the increase in M_w. These interaction sites may result from the aromatic rings forming aromatic ring stacking or the hydroxyl groups forming hydrogen bonds, depending on the tested polyphenol and protein, as previously proposed for other enzymes (*18, 29, 30*).

In order to study the mechanism involved in the inhibition of PL by procyanidins, physicochemical methods such as fluorescence and light scattering techniques were employed.

Fluorescence Quenching. The inhibition of PL by procyanidins is not by itself indicative that these compounds are interacting with the enzyme. It solely indicates that the hydrolysis kinetic is being affected by these compounds. Intrinsic fluorescence quenching of the tryptophan residue was used to elucidate this feature. Porcine pancreatic lipase (PL) consists of 448 amino acid residues in a single polypeptide of globular shape containing seven tryptophans with intrinsic fluorescence (Trp 17, Trp 30, Trp 86, Trp 107, Trp 253, Trp 339, and Trp 403) (*31*).

Changes in the emission spectrum of tryptophan are common in response to structural modifications of proteins (*22*). The observed λ_{em} for lipase ($\lambda_{em} = 362$ nm) is higher that the λ_{em} for the isolate indole group of tryptophan ($\lambda_{em} = 340$ nm) (**Figure 1**). This shift to longer wavelengths (red shift) indicates that the fluorescing residues in this protein are located on the surface of the molecule and have contact with solvent molecules (*22*).

The addition of procyanidins to lipase caused a reduction of protein fluorescence intensity ($\lambda_{em} = 362$ nm) known as quenching effect (**Figure 1**). On the other hand, as it did not alter considerably
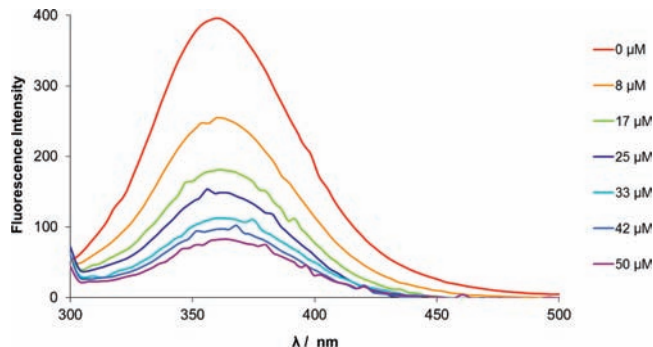

Figure 1. Fluorescence emission spectra (at $\lambda_{ex} = 290$ nm) of PL (2.5 μM) in the presence of increasing concentrations of procyanidin fraction III. Each curve represents a triplicate assay after correction for polyphenol fluorescence.

the shape of protein spectra, it was assumed that the interaction between the enzyme and procyanidins did not significantly change the environment of the seven tryptophan residues and consequently the enzyme structure (*24*).

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 2A**). In the experimental conditions described herein, this plot for FI and FII 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 quenching (complex forming) and dynamic quenching (collision induced), or it could mean the existence of a "sphere of action" (*24*). In this case the Stern−Volmer plot is linearized by representing $\ln(F_0/F)$ vs [quencher] (**Figure 2B**), and the slope of this plot corresponds to the apparent static quenching constant (K_{app}).

From the analysis of **Figure 2** and **Table 2**, it is clear that an increase in the ability to quench the fluorescence of lipase occurs as the average degree of polymerization increases. The apparent static constants indicate that the interaction between procyanidins and lipase is stronger for the more polymerized procyanidin fractions. In order to determine if any specific interaction occurs between lipase (fluorophore) and procyanidins (quencher), the apparent bimolecular quenching constant (k_q^{app}) was calculated. This is done by dividing K_{app} by the lifetime of the fluorophore in the absence of quencher (τ_0) (**Table 2**). The maximum value possible for diffusion-limited quenching (dynamic mechanism) in a water medium 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 (*32, 33*). The results obtained with these procyanidin fractions indicate that the mechanism by which procyanidins associate with lipase is of a static type involving a stable interaction between the two.

To estimate the stoichiometry of the aggregation process between procyanidin/PL, the changes in fluorescence intensities when tannins are added to the protein solution were monitored. The fluorescence intensity for the procyanidin titration (at constant
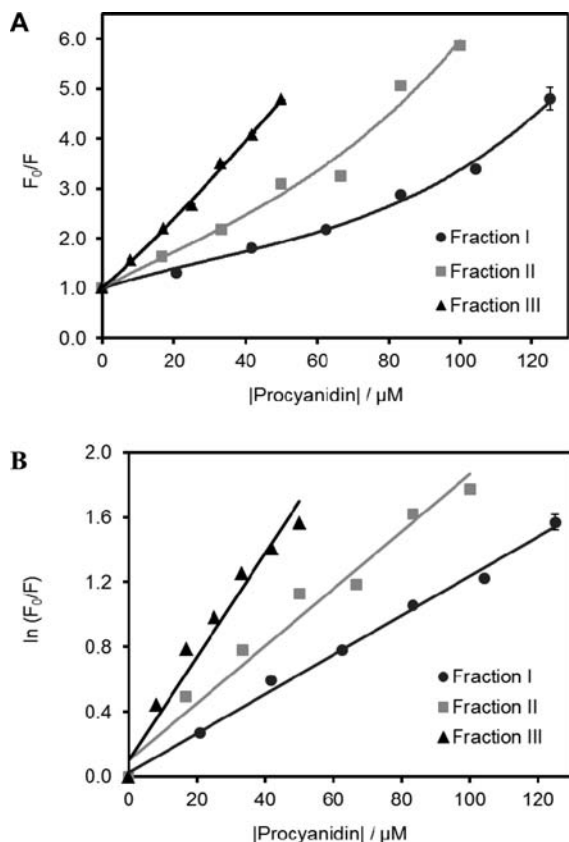
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A

B

Figure 2. Stern–Volmer (**A**) and modified Stern–Volmer (**B**) plot describing tryptophan quenching of pancreatic lipase (2.5 μM) at pH 7.0 by increasing concentrations of oligomeric procyanidin fractions (●, FI; gray □, FII; ▲, FIII). The fluorescence emission intensity was recorded at λ_{ex} 290 nm, and the λ_{em} maximum occurred at 362 nm.

protein concentration) can be fitted using eq 3 describing the binding of tannins at multiple sites (*n*) on the protein (**Figure 3**). K_d, I_{Fmax}, and *n* values for tested procyanidin fractions may then be determined and are reported in **Table 3**. The increasing M_w of procyanidin fractions influences not only K_d but also *n*. It is observable that as the degree of polymerization increases, there is a decrease in the dissociation constant that is indicative of a stronger affinity between procyanidins and PL. Interestingly, there is also a decrease in the number of binding sites that are involved in the interaction. Assuming that a protein molecule possesses a fixed number of binding sites for PC, we may be interpret this decrease as resulting from each molecule of higher M_w procyanidins occupying a larger number of binding sites in the protein.

Light Scattering of PL/Procyanidin Solutions. Light scattering studies were conducted in order to gain information at macromolecular level regarding the interaction of procyanidins with PL. The average size of PL/procyanidin aggregates in solution was measured using dynamic light scattering (DLS). The DLS device measures fluctuations in the intensity of scattered light to calculate 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) or the most abundant particles (number). In the present case it is useful to use the number distribution since the most abundant particles in solution are expected to be the formed aggregates.

The plot of relative size vs procyanidin concentration reveals an increase in size with increasing concentration of procyanidin fractions up to a maximum that depends on the nature of
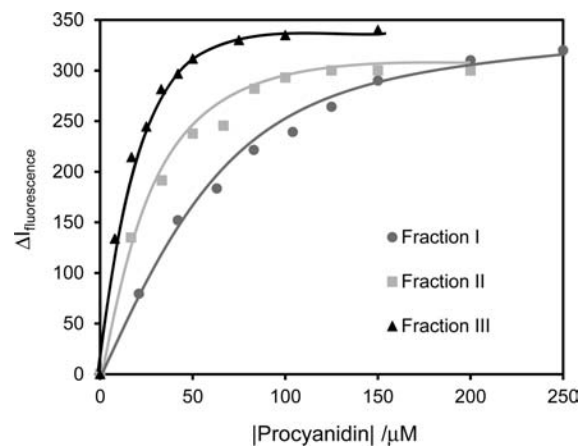

Figure 3. Comparison of observed (symbols) and fitted (lines) fluorescence intensities with increasing concentrations of oligomeric procyanidin fractions (●, FI; gray □, FII; ▲, FIII) using the fitting parameters represented in eq 3. PL concentration was fixed at 2.5 μM.

Table 3. Dissociation Constant (K_d), Number of Binding Sites (*n*), and Difference between Protein Fluorescence Alone and Saturated with Tannins (I_{Fmax}) for the Interaction between Oligomeric Procyanidin Fractions and PL Determined by Fluorescence Quenching Assuming a Multisite Binding Model (eq 3)[a]

	K_d (μM)	*n*	I_{Fmax}
fraction I	44.7 ± 7 a	17.0 ± 0.5 a	386 ± 21 a
fraction II	12.9 ± 0.7 b	10.1 ± 0.4 b	336 ± 1 ab
fraction III	6.4 ± 0.9 c	4.8 ± 0.8 c	359 ± 5 b

[a] Values with different superscript letters are significantly different (*P* < 0.05).

procyanidin (**Figure 4A,B**). After this maximum the size of aggregates begins to decrease. The more polymerized procyanidin fraction reaches this maximum at lower procyanidin concentration. It was also shown that although the concentration at which the maximum size of aggregates occurs depends on the molecular weight of the procyanidins, the same is not true for the actual size of aggregates (**Figure 4B**). In fact, the largest aggregates were formed with fraction II, ca. 900 nm, while fractions I and III formed the maximum size aggregates at 200 and 500 nm, respectively.

Through DLS it is also possible to measure the amount of light scattered by the particles in solution (per second) that depends on both the size and number of aggregates in solution. The representation of count rate vs procyanidin concentration (**Figure 4C**) is similar to the behavior described for aggregate size. An increase in the count rate is observed with increasing procyanidin concentrations up to a maximum that occurs near the same concentration as for maximum size. This maximum of intensity may be explained by the increase of the size of aggregates that leads to more scattering of light.

The decrease in light scattering observed after the maximum is usually explained by a model in which excessive procyanidin concentration leads to the formation of aggregates of smaller sizes that reduce the overall scattered light (*34*). This model states that at high concentrations the protein is fully covered by tannins, and therefore the tannin molecules are unable to bridge protein molecules and cause aggregation. The decrease in scattered light (count rate) accompanied by a decrease in the size of aggregates indicates that the previously formed aggregates are suffering a variation of stoichiometry that induces less scattering of light.

An additional technique based on the measurement of light scattered at 90° (nephelometry) was employed to evaluate the evolution of lipase/procyanidin aggregation with the increase in
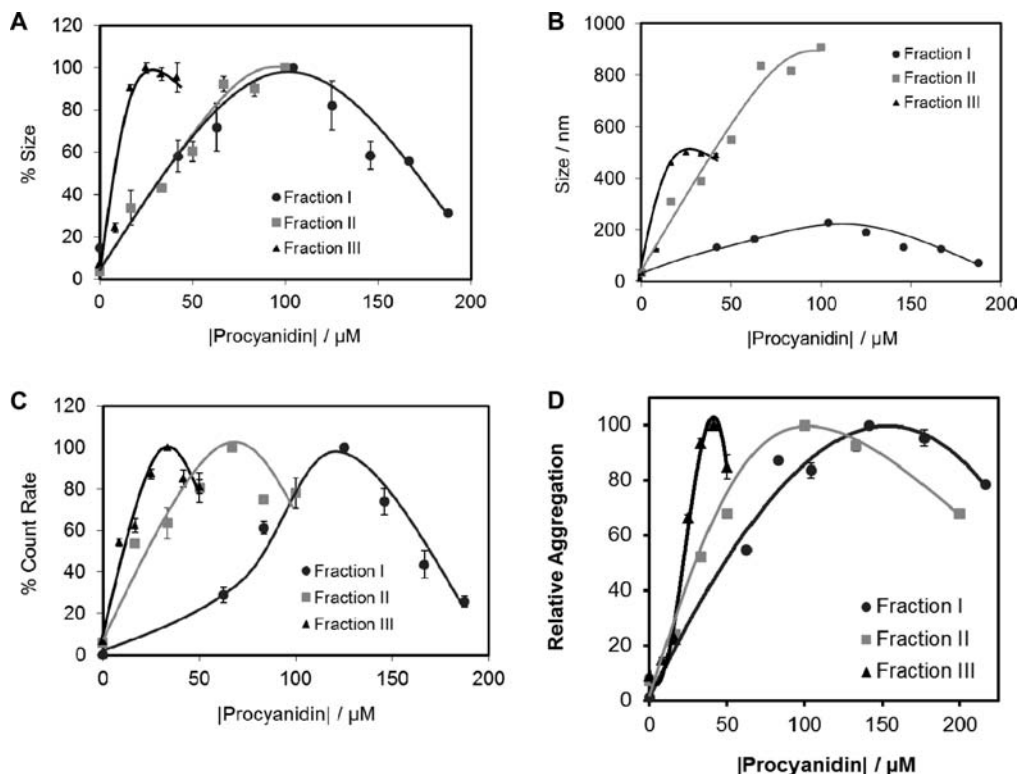
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Figure 4. Changes in PL (2.5 μM)/procyanidin percent aggregate size (**A**), absolute aggregate size (**B**), and aggregation intensity [count rate (**C**) and nephelometry (**D**)] by increasing concentrations of oligomeric procyanidin fractions (●, FI; gray □, FII; ▲, FIII) at pH 7.0.

procyanidin concentration (**Figure 4D**). As expected and since the obtained parameter corresponds to the scattering of light in solution, a behavior similar to the DLS count rate measurement was observed.

In conclusion, the interaction process evaluated by fluorescence quenching, dynamic light scattering, and nephelometry appears however to occur at concentrations much smaller than the effective enzymatic activity inhibition. Indeed, the maximum of light scattering and aggregate size occur for all fractions at lower procyanidin concentrations than the determined IC$_{50}$, indicating that the effect of the light scattering phenomena in enzyme inhibition is small. It seems that before enzymatic inhibition can occur there is aggregation between PL and procyanidins and may occur only at very high stoichiometric ratios between procyanidins/enzyme, i.e., when PL molecules are to a large extent covered with procyanidin.

In the digestive system the inhibition of PL may contribute to a reduction of the hydrolysis of triacylglycerols, contributing to a reduced absorption of fatty acids from the diet. One factor that may influence the effective inhibitory power observed *in vivo* is the metabolism of procyanidins in the digestive system. Even though procyanidins may be metabolized in the digestive tract, this is likely to occur in the large intestine where pancreatic lipase is already inactive (*16*). It is therefore believed that in the initial portion of the small intestine polymeric procyanidins should have suffered very little modification. The result obtained may be relevant to the development of new lipase inhibitors for therapeutic purposes and also to assess the health benefits of procyanidin-rich (or enriched) foods and beverages. This work increases also the knowledge on the antinutritional properties of procyanidin-containing foods.

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