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ORIGINAL PAPER

Fluorescence Quenching Study on the Interaction between Quercetin and Lipoxygenase

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Abstract The interaction between quercetin and lipoxygenase was investigated by fluorescence spectroscopy. The analysis of the emission quenching at different temperatures revealed that the quenching mechanism correspond to a static process and, as consequence, a complex quercetin-lipoxygenase is formed. The thermodynamic parameters ΔG, ΔH and ΔS were calculated to be−32.57 kJmol^{-1}, −3.21 kJmol^{-1} and 87.14 Jmol^{-1}K^{-1} respectively, which suggest that hydrophobic forces plays a major role in the stabilization of the complex quercetin-lipoxygenase. The distance, r, between donor (lipoxygenase) and acceptor (quercetin) was calculated to be 3.84 nm based on Förster's non-radiative energy transfer theory. The results obtained from the evaluation of three dimensional florescence spectra suggest a conformational modification of the protein in the region of the coupling with quercetin.

Keywords Quercetin · Lipoxygenase · Flavonoids · Fluorescence · Antioxidants

Introduction

Flavonoids are a group of plant metabolites with more than 6,000 different compounds, being flavonols an important subclass. The major representative compound of this subclass is quercetin (3,3′,4′,5,7-pentahydroxyflavone) a typical polyphenolic compound found in a variety of fruits and vegetables. A number of studies showing its biological properties including antioxidative, anti-inflammatory and cancer preventive effects, indicate that quercetin is potentially beneficial for human health [1]. These effects have been attributed to various mechanisms mediated by the inhibition of enzymes that activate carcinogens, the modification of signal transduction pathways and the interaction with receptors and other proteins [2]. The biological properties described for flavonoids are directly related which processes as stress oxidative, carcinogenesis or inflammation, in which is involved the prooxidante enzyme lipoxygenase. Lipoxygenase are a class of iron containing enzymes that catalyzes the incorporation of dioxygen into polyunsaturated fatty acids, giving rise to respective hydroperoxy/hydroxyl fatty acid derivative. In mammals is the key enzyme in leukotriene biosynthesis, a group of highly potent molecules that mediate inflammatory and allergic reactions, being involved in various disease conditions such as asthma, allergic rhinitis or psoriasis. Due to the contribution of eicosanoids to the pathogenesis of inflammatory and allergic diseases, lipoxygenase pathway is a target for the development of antiinflammatory agents [3].

It has been reported that quercetin is an efficient inhibitor of lipoxygenase with a potential similar to that synthetic inhibitor phenidone or NDGA [4–6]. The study of the interaction of flavonoids with lipoxygenase merits particular attention from a pharmacological point of view, since this prooxidante enzyme is a potential target for the beneficial effects of flavonoids, however the mode of interaction between flavonoids and lipoxygenases have scarcely been studied. In previous work we reported by semiempirical studies that the interaction of quercetin with lipoxygenase is related to the spatial adaptation of the flavonoid to the hydrophobic cavity that constitutes the channel of access of substrate to the catalytic site [7].

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With the aim to obtain a more complete information about the interaction quercetin – lipoxygenase, we used in this study fluorescence spectroscopy to investigate the characteristics of the binding flavonoid-enzyme. The fluorescence quenching is the methodological approach more adequate to investigate the interaction of ligands with proteins and for the measurement of binding affinities [8]. The fluorescence intensity of a fluorophore can be decrease by a colisional process (dynamic quenching) and/or by the formation of a complex between quencher and fluorophore (static quenching). The intrinsic fluorescence of proteins is produced by Trp and Tyr when are excited at 280 nm. The evaluation of the quenching of this fluorescence gives valuable information about the accessibility of ligands (quencher) to proteins (fluorophore) [9]. The study of intrinsic fluorescence extinction of proteins has been used to analyze the binding of flavonoids to proteins as BSA [10] or catalase [11].

Also, it has been reported that lipoxygenase possesses a fluorescence intrinsic produced by aromatic aminoacids when are excited at 280 nm. From the study of this fluorescence information it has been obtained information about alterations of molecular properties [12], conformational transitions [13] or spectroscopic characterization [14] of lipoxygenase. In this study it has been investigated the characteristics of the interaction between quercetin and lipoxygenase using fluorescence spectroscopy. From the evaluation of the quenching of the intrinsic fluorescence of lipoxygenase at various temperatures were determined the binding constants and the nature of the binding forces. In addition, the determination of the Förster energy transference between lipoxygenase and quercetin gave information about the distance between the fluorophore and quercetin in the site of interaction with the enzyme. Finally, the use of three dimensional spectra fluorescence has allowed us to check a possible modification in the secondary structure of the enzyme.

Experimental

Reagents

Quercetin, linoleic acid and lipoxygenase from soybean (type V) were from Sigma (Madrid, Spain). All general reagents were analytical grade.

Apparatus and Measurements

All fluorescence measurements were made with a Varian Cary spectrofluorimeter equipped with thermostatization system and temperature controller, using 5.0 nm excitation and 5.0 nm emission slit widths. A UV–vis 1603 Shimadzu spectrophotometer equipped with a 1.0 cm quartz cells was used for scanning of the UV spectrum.

Lipoxygenase activity was measured spectrophotometrically at 234 nm as in [5] using 15 mUnits of enzyme in the presence of 100 μM linoleic acid in 50 mM potassium phosphate buffer, pH 7.5.

A stock solution of quercetin was prepared in methanol (the effect of methanol on fluorescence measurements was checked and was considered as negligible). For each scan 2 μL of quercetin solution was added to 3 mL lipoxygenase solution to give a final concentration in the range 0.8 to 4.0 μM. In all the measurements the effect of dilution was corrected, each measurement was repeated by triplicate and the mean and S.D. was determinate. Statistically significant differences between the parameters obtained from the fluorescence quenching measurements were determined using ANOVA with a 95% confidence limits ($p<0.05$).

Fluorescence quenching spectra of lipoxygenase were obtained using a λ_{ex}=280 nm and a λ_{em}=290–450 nm. Appropriate blanks corresponding to the buffer were subtracted to correct background fluorescence.

To evaluate the inner filter effect, absorbance measurements were performed at excitation and emission wavelength of lipoxygenase. Observed fluorescence values were corrected using the equation [9]:

$$F_{corr} = F_{obsd} e^{\frac{A_{280}+A_{333}}{2}}$$

Where A_{280} and A_{333} are the absorbance of protein and ligands at excitation and emission wavelength respectively.

Results and Discussion

Lipoxygenase Inhibition by Quercetin

When the system lipoxygenase-linoleic acid is incubated in the presence of quercetin at pH 7.5, an inhibitory effect concentration dependent is detected. This effect shows the characteristics of a competitive mechanism as can be deduced from Lineweaver-Burk plot (Fig. 1). The value of *Ki* for quercetin inhibition (3.73 μM), reveal the high efficiency of this polyphenol as lipoxygenase inhibitor. In previous work [7] we showed that the high efficiency of quercetin as inhibitor is related with his structural and electronic properties, responsible for a favorable interaction with the binding site in the lipoxygenase molecule. The competitive inhibition produced by quercetin on lipoxygenase activity suggest that linoleic acid is displaced from the active site and that, as consequence, the binding site should be located near, or at, lipoxygenase catalytic site. May be of pharmacological interest to know the characteristics of the interaction site of lipoxygenase with quercetin. With this

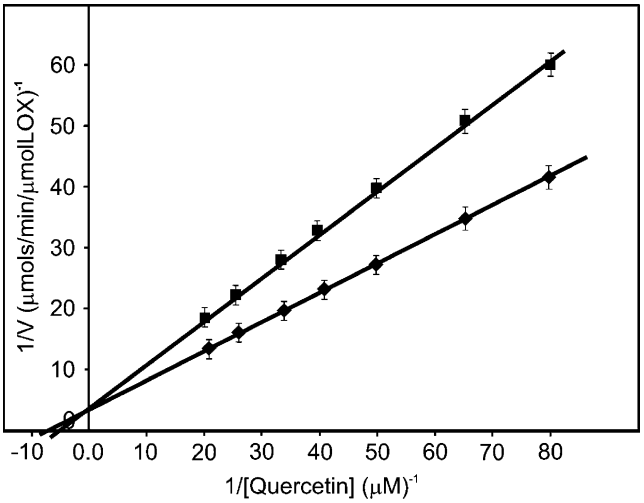

Fig. 1 Lineweaver-Burk plot of lipoxygenase-linoleic acid system in absence (*rhombus*) and presence (*squares*) of 3.7 μM quercetin at pH 7.5

aim, the type of binding forces, the number of interaction points, and the possible modification in the microenvironment of the protein at the quercetin binding site, were investigated. To obtain this information we studied the quenching of intrinsic fluorescence of lipoxygenase by quercetin.

Binding Constants and Number of Binding Sites

The existence of interaction between lipoxygenase and quercetin was established by UV–vis absorption spectrometry. Figure 2 shows the UV–vis absorption spectra of lipoxygenase (A) and quercetin (B). Plot C is the difference between the absorption spectrum of the mixture lipoxygenase-quercetin and the spectrum of quercetin at the same concentration (2 μM). Our data clearly shows that plot C is under the plot corresponding to lipoxygenase, indicating that there are binding interactions between lipoxygenase and quercetin [15].

Fluorescence Quenching

Fluorescence spectroscopy has been usually used in the study of molecular interactions between ligands and proteins, mainly due to the high sensitivity of this methodological approach and the variety of parameters related with the interaction molecular that can be obtained.

To obtain detailed information about the interaction lipoxygenase-quercetin, quenching fluorescence measurements were carried out.

Figure 3 shows the emission spectra of lipoxygenase in the presence of quercetin concentrations ranging from 0.0 to 4.0 μM. When lipoxygenase is excited at 280 nm we obtained a broad emission band centered at 330–340 nm produced by the emission of Tyr and Trp residues [16]. It can be seen from Fig. 3 that increasing concentrations of quercetin leads to a regular quenching of lipoxygenase fluorescence without modification of the maximum of emission.

Quenching of fluorescence can occur by two different mechanisms classified as static or dynamic quenching. These mechanisms can be distinguished by their differing dependence on temperature. For static mechanism the quenching rate constant decreased with the increased temperature and the opposite effect is observed for the case of dynamic quenching.

With the aim to determine the type of mechanism, lipoxygenase fluorescence data were plotted, as relative fluorescence intensity (F_0/F), against quercetin concentra-

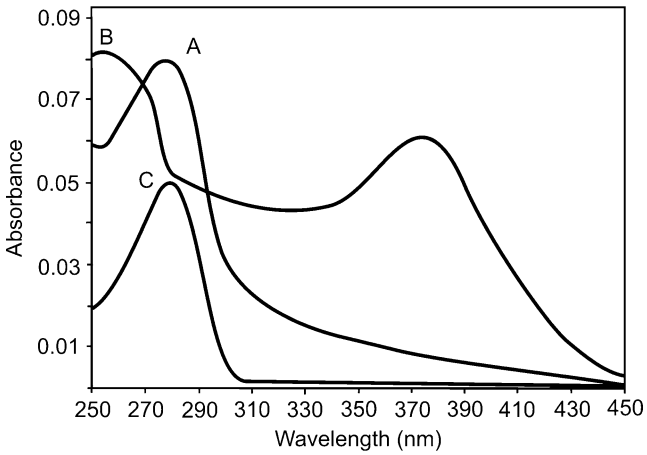

Fig. 2 UV–vis absorption spectrum of lipoxygenase (**a**) and quercetin (**b**). Plot C is the difference between the spectrum of the mixture lipoxygenase-quercetin and the spectrum of quercetin at the same concentration (2 μM)

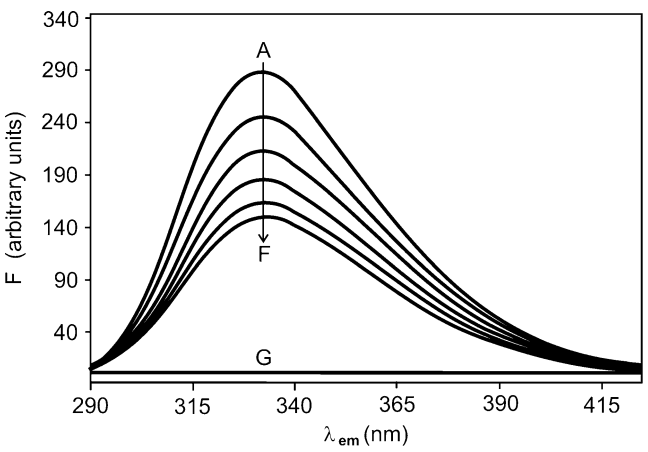

Fig. 3 Emission spectra of lipoxygenase (λ_{exc}=280 nm) in the presence of quercetin at 0.0; 0.8; 1.6; 2.4; 3.2 and 4.0 μM (plots A to F respectively). Plot G correspond to the emission spectrum of quercetin at the same λ_{exc}. All measurements were carried out at 296 K

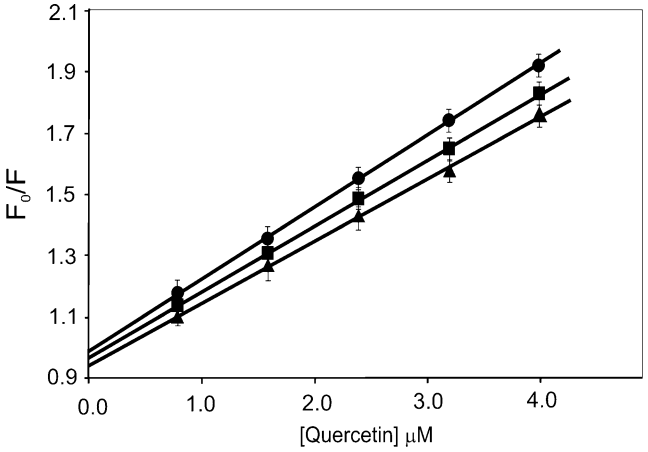

Fig. 4 Stern-Volmer plot for the lipoxygenase fluorescence quenching caused by quercetin at 296 K (*rhombus*), 303 K (*squares*) and 310 K (*triangles*). In all cases λ_{exc}=280 nm and λ_{em}=333 nm

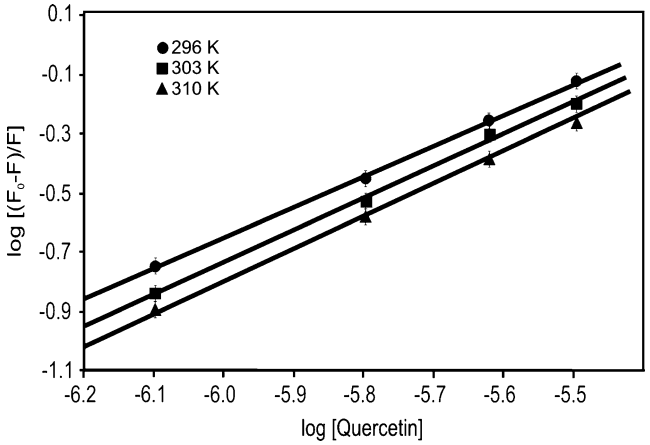

Fig. 5 Plot of log [(F_0-F)/F]=log K_B+n log [Q] for determination of binding parameters at indicated temperatures. Other conditions are the same than in Fig. 3

tion, being F and F_0 the fluorescence intensity in the presence and absence of quercetin respectively. The quenching constants were obtained from the Stern-Volmer plot by linear regression using the expression:

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

where k_q is the bimolecular quenching constant, K_{SV} the Stern-Volmer quenching constant and τ_0 is the lifetime of the fluorophore in the absence of quencher being equal to 10^{-8} s [9].

The possible mechanism can be deduced from the Stern-Volmer plots (1) obtained at different temperatures (296 K, 303 K and 310 K) as is shown in Fig. 4. From the experimental data were obtained the following equations by liner regression fitting: $F_0/F = 2.35 \times 10^5[Q] + 0.9878$ at 296 K, $F_0/F = 2.10 \times 10^5[Q] + 0.9696$ at 303 K and $F_0/F = 2.02 \times 10^5[Q] + 0.9767$ at 310 K. According with these data the K_{SV} and k_q obtained at each temperature are indicated in Table 1. It is known that the maximum scatter collision quenching constant for a dynamic mechanism is 2.0×10^{10} $Lmol^{-1}s^{-1}$[8]. The rate constants obtained for lipoxygenase quenching caused by quercetin is greater than rate constant limit for dynamic quenching and, as consequence, with great probability the mechanism for the quenching caused by quercetin is of static type. In addition, the K_{SV} decreased with the temperature rising, a characteristic for static quenching, due to that high temperatures result in the dissociation of complexes formed [9].

The results obtained suggest the formation of a nonfluorescent lipoxygenase-quercetin complex. If we assume that there are n possible binding sites for quercetin (Q) on lipoxygenase (L), the reaction for complex formation may be:

$$nQ + L \leftrightarrow Q_nL \quad (2)$$

Being the binding constant K_B:

$$K_B = \frac{[Q_nL]}{[Q]^n[L]} \quad (3)$$

Where [Q], [L] and [Q_nL] are the quercetin, lipoxygenase and complex concentrations respectively. If total lipoxygenase concentration is [L_0], then [Q_nL] = [L_0]-[L] and by substitution in (3) we can obtain the expression (4):

$$K_B = \frac{[L_o] - [L]}{[Q]^n[L]} \quad (4)$$

Table 1 Stern-Volmer quenching constants of LOX-RESV system at different temperatures

T (K)	Ksv ($Lmol^{-1}$)	kq ($Lmol^{-1}s^{-1}$)	R[a]	SD
296	2.35×10^5	2.35×10^{13}	0.998	0.019
303	2.10×10^5	2.10×10^{13}	0.994	0.017
310	2.02×10^5	2.02×10^{13}	0.977	0.011

[a] Regression coefficient

Table 2 Binding constant (K_B) and binding sites (n) at different temperatures

T(K)	$K_B\times10^5$ ($Lmol^{-1}$)	n	R[a]
296	6.61	1.10	0.999
303	6.17	1.09	0.995
310	3.47	1.03	0.992

[a] Regression coefficient

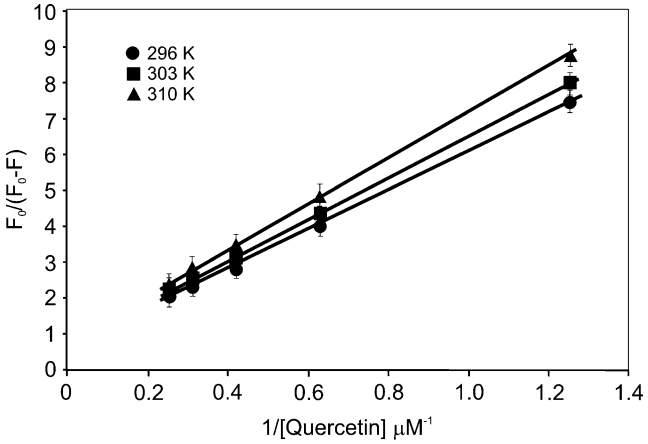

Fig. 6 Modified Stern-Volmer plot for the system quercetin-lipoxygenase at 296 K (*rhombus*), 302 K (*squares*) and 310 K (*triangles*)

If we consider that fluorescence intensity of lipoxygenase is proportional to his concentration, we can write Eq. (5):

$$\frac{[L]}{[L_o]} = \frac{F}{F_o} \tag{5}$$

and by substitution in (4) we obtain the expression (6)[17]:

$$log\frac{(F_o - F)}{F} = logK_B + nlog[Q] \tag{6}$$

The values for K_B and n can be obtained from (6), plotting log [(F_0-F)/F] vs log[Q] and determining the intercept and slope as is shown in Fig. 5. The results obtained from this plot are presented in Table 2. In the range of temperature studied, the value obtained for n, near to 1, indicates that the binding of quercetin to lipoxygenase is in a ratio 1:1. From the K_B value it can be deduced the formation of a complex relatively stable, only sensible to higher temperatures assayed.

Determination of Forces Responsible for the Formation of the Complex

Our data clearly shows a dependence of binding constants on temperature and therefore the evaluation of thermodynamic parameters can give information about the acting forces between lipoxygenase and quercetin. These forces include mainly hydrogen bond and van der Waals, electrostatics and hydrophobic forces. The mode of interaction may be investigated by evaluation of the magnitude and signs of thermodynamic parameters enthalpy ΔH, entropy ΔS and free energy change ΔG.

The enthalpy and entropy changes can be evaluated according to the van't Hoff Eq. (7):

$$lnK = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \tag{7}$$

In the case that fluorescence quenching is produced by a complex protein- quencher, K can be obtained from the modified Stern-Volmer equation.

$$\frac{F_o}{F_o - F} = \frac{1}{faK}\cdot\frac{1}{[Q]} + \frac{1}{fa} \tag{8}$$

Where f_a is the fraction of accessible fluorescence and K is the effective quenching constant for the accessible fluorophore, analogous to the Stern-Volmer constant at the corresponding temperature.

The free energy change is estimated from the expression (9):

$$\Delta G = \Delta H - T\Delta S \tag{9}$$

Figure 6 was obtained by plotting the modified Stern-Volmer Eq. (8) at three different temperatures and K values are shown in the Table 3. The values of ΔH and ΔS also shown in Table 3, were obtained from the van't Hoff plot (Fig. 7). It is to be noted the negative value for ΔG, meaning the spontaneous character of the process. The negative enthalpy suggests that hydrogen bonds or van der Waals forces may be involved in the binding, but the low value obtained suggest that the contribution of these forces may be negligible. In contrast, the high positive value of ΔS is an evidence for the participation of hydrophobic interactions in the binding of quercetin to lipoxygenase [18].

Binding Distance between Quercetin and Lipoxygenase

The Förster non radiative energy transference can be used for determination of the distance between a donor of fluorescence (Tyr and Trp of lipoxygenase) and an acceptor (quercetin). This transference only is possible if the fluorescence emission spectrum of donor overlap the UV absorption spectrum of acceptor. Figure 8 shows the overlap of the UV absorption spectrum of quercetin with

Table 3 Modified Stern-Volmer association constants and related thermodynamic parameters

T(K)	K ($Lmol^{-1}$)	R^a	ΔH ($kJmol^{-1}$)	ΔG ($kJmol^{-1}$)	ΔS ($Jmol^{-1}K^{-1}$)	R^b
296	1.31×10^5	0.979		−31.90		
303	1.27×10^5	0.998	−3.21	−32.57	87.14	0.999
310	1.24×10^5	0.998		−33.24		

[a] Regression coefficient of K values

[b] Regression coefficient of van´t Hoff plot

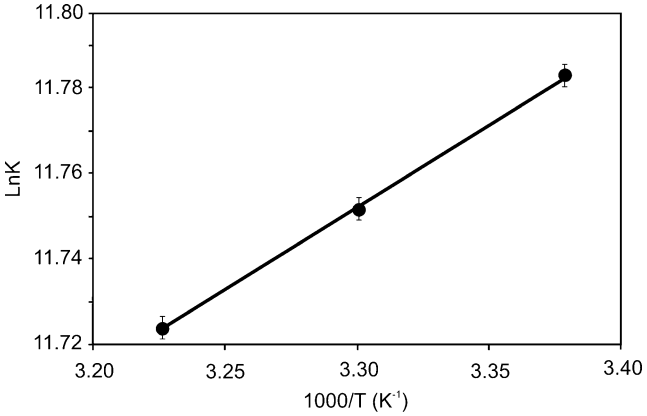

Fig. 7 van't Hoff plot for the binding of quercetin to lipoxygenase

the fluorescence emission spectrum of lipoxygenase. The efficiency of energy transference was calculated according Förster energy transference theory [9] using the Eq. (10),

$$E = \frac{R_o^6}{R_o^6 + r^6} = 1 - \frac{F}{F_o} \tag{10}$$

where F and F_0 are the fluorescence intensity of lipoxygenase in the presence and absence of quercetin, r is the distance between donor and acceptor and R_0 is the distance at which the efficiency of the transfer is a 50%, being calculated from the Eq. (11),

$$R_o^6 = 8.79 \times 10^{-25} K^2 n^{-4} \varnothing J \tag{11}$$

where K^2 is the spatial orientation factor of dipole, n is the refractive index of the medium, $\varnothing$ is the fluorescence quantum yield of the donor and J is the spectral overlap between donor emission and acceptor absorption, being obtained from the Eq. (12),

$$J = \frac{\int_o^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 d(\lambda)}{\int_o^\infty F(\lambda) d\lambda} \tag{12}$$

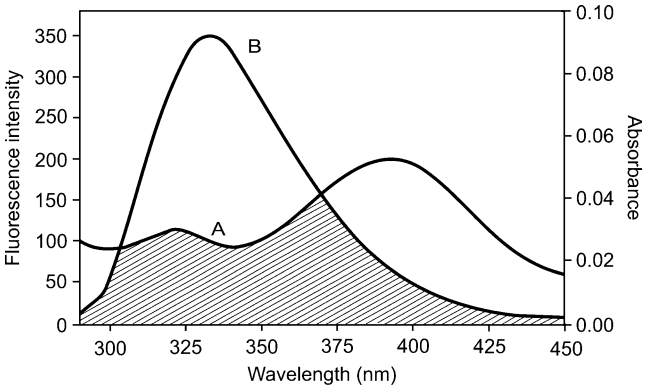

Fig. 8 Spectral overlap of the UV spectrum of quercetin (**a**) with the emission spectrum of lipoxygenase (**b**). Both spectrum where carried out at a concentration of 2 μM

being $F(\lambda)$ the fluorescence intensity of donor at the wavelength λ, $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at the wavelength λ. Data for the determination of J were obtained from the spectra of Fig. 8. In the present study it has been assumed that K^2=2/3, n=1 and ϕ=0.15 [8]. From our experimental data we determined that R_0= 2.88 nm and r=3.84 nm. The donor-acceptor distance, r, possesses a value in the range 2–8 nm, indicating that the energy transfer from lipoxygenase to quercetin occurs with high possibility and that exist an interaction with formation of a complex donor-acceptor responsible for the static quenching.

Three Dimensional Fluorescence Spectroscopy

Three-dimensional fluorescence spectroscopy can inform about conformational changes of lipoxygenase by the

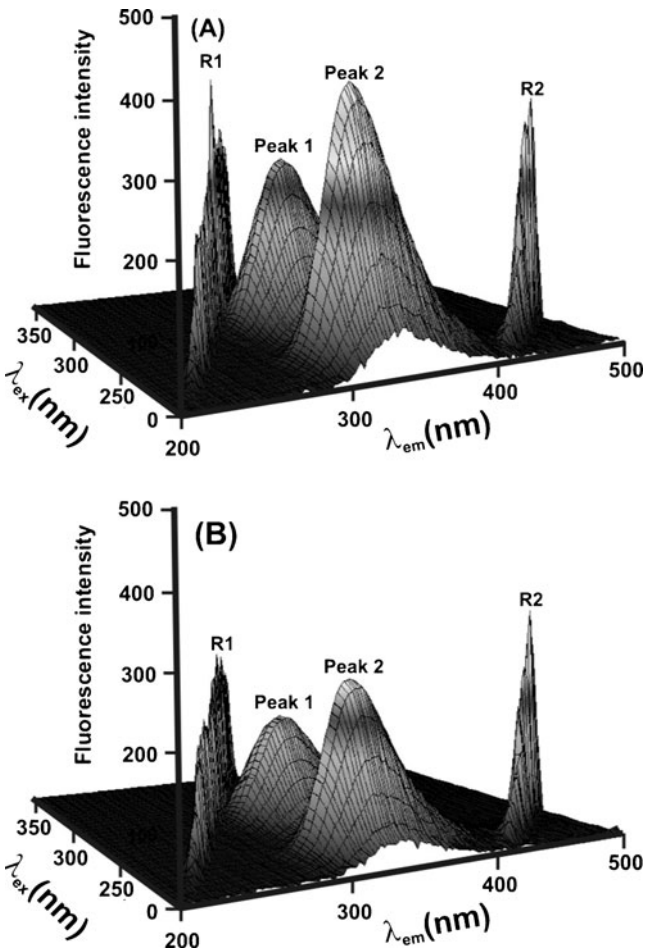

Fig. 9 Three dimensional fluorescence spectra of lipoxygenase (plot A) and of the system lipoxygenase-quercetin (plot B). Concentration of lipoxygenase in both spectra was 2 μM and quercetin concentration used in plot B was 4 μM. R_1 and R_2 peaks corresponds to Rayleigh scattering

interaction with quercetin. Figure 9 shows the three dimensional fluorescence spectra of lipoxygenase in the absence (A) and in the presence (B) of quercetin. Peak 1, centered at λexc=280 nm/λem=333 nm mainly reveals the behavior of Trp and Tyr residues and possesses a value for fluorescence intensity of 271.1 units for lipoxygenase in absence of quercetin (peak 1, plot A), this value decreased to 173.6 units (a 37%) in the presence of quercetin. The second peak centered at λexc=229 nm/λem=333 nm mainly exhibits the spectral characteristics of the microenvironment of polypeptide backbone structure, being related the modification of this fluorescence intensity with modifications of secondary structure of protein [18, 19]. The florescence intensity for this peak was 413.1 units for lipoxygenase in absence of quercetin (peak 2, plot A), decreasing to 258.8 units (a 37%) in the presence of quercetin. The decrease of fluorescence intensity of lipoxygenase for both peaks indicated that the interaction with quercetin may induce a slight conformational modification of protein in the microenvironment of the binding site.

The results obtained by fluorescence spectroscopy indicating that quercetin forms a complex with lipoxygenase, near to catalytic site, in a spontaneous process in which hydrophobic interactions are involved, fit well with the known structure of the access channel for the substrate to the catalytic site of lipoxygenase. This region, principally composed by hydrophobic aminoacids (Trp340, Phe346, Gly353, Asn355, Lys483, Asp490, His494, His499, Ala542, Leu546, Glu697, Asn706 y Leu754) [20], is highly conserved for lipoxygenases of animal and vegetal sources and contains a Trp residue that, with high probability, is the main fluorophore residue responsible for the quenching of fluorescence produced by quercetin.

Conclusions

The binding interaction of quercetin with the prooxidant enzyme lipoxygenase was studied by fluorescence spectroscopy. The results obtained suggest that, on the basis of the competitive inhibition detected, the interaction should be located near or at the catalytic site. The fluorescence quenching study clearly reveals a static mechanism and the formation of a complex between quercetin and lipoxygenase. The thermodynamic parameters obtained from van't Hoff equation indicate that hydrogen bonds and hydrophobic interactions are the main forces responsible for the stabilization of the complex spontaneously formed. The binding distance, r, between quercetin and lipoxygenase was determined according to the Förster non radiative energy transfer theory and the value obtained confirms the formation of a stable complex. Finally, the results obtained from three dimensional fluorescence spectra show that binding of quercetin to lipoxygenase can induce conformational changes in the secondary structure of the enzyme.

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