Proceedings of ECOpole
Vol. 3, No. 1 2009

Lucie TRNKOVÁ[1,2], Iva BOUŠOVÁ[2], Lucie RYŠÁNKOVÁ[1], Petra VRABCOVÁ[1] and Jaroslav DRŠATA[1,2]

ANTIOXIDANTS AND ENVIRONMENTAL STRESS: SPECTROSCOPIC STUDY ON STABILITY OF NATURAL COMPOUNDS AND THEIR INTERACTION WITH A MOLECULE OF PROTEIN IN AN *IN VITRO* MODEL

ANTYOKSYDANTY A STRES ŚRODOWISKOWY, BADANIA SPEKTRALNE STABILNOŚCI SUBSTANCJI NATURALNYCH I ICH ODDZIAŁYWANIE Z MOLEKUŁĄ BIAŁKA

Abstract: The stability of eight hydroxycinnamic acids (HCAs) during long-term incubation under physiological conditions was studied by UV-VIS absorption spectroscopy and their possibility of binding to a model protein (bovine serum albumin, BSA) under physiological conditions was investigated by tryptophan fluorescence quenching method. The obtained results suggest that the stability of hydroxycinnamic acids is dependent upon its individual structure and duration of incubation. The monosubstituted derivatives (coumaric acids) were stable within the course of long-term incubation, while di- and trisubstituted derivatives decomposed easily. It was found out that all studied compounds changed fluorescence emission spectrum of BSA. The Stern-Volmer analysis was employed in order to explore binding of HCAs to BSA in details. The binding constants (K_b), number of binding sites (n) and the free energy changes (ΔG^0) were determined. The binding affinity was strongest for rosmarinic acid and ranked in the following order rosmarinic acid > chlorogenic acid > sinapic acid > caffeic acid > ferulic acid > *o*-coumaric acid > *p*-coumaric acid > *m*-coumaric acid. All free energy changes (ΔG^0) possessed negative sign indicating the spontaneity of HCAs binding to BSA.

Keywords: oxidative stress, antioxidant, hydroxycinnamic acid, serum albumin, protein-ligand binding

There is a large amount of air pollutants in our environment and many of them show adverse oxidative effects on living organisms [1]. Although the properties of air pollutants vary markedly, they all have one common feature. They can cause oxidative stress, a state in which the prooxidant-antioxidant balance is seriously impaired. This imbalance can occur when the generation of reactive oxygen species overwhelms endogenous antioxidant systems. Oxidative stress can have serious impact on human health [1, 2]. Recently, considerable attention has been focused on the study of naturally occurring compounds with antioxidant effects (eg flavonoids, hydroxycinnamic acids, carotenoids) which can protect body against environmental pollutants. Hydroxycinnamic acids (HCAs) are natural compounds widely distributed in higher plants and are used in folk medicine because of their antioxidant and other effects [3, 4]. Serum albumin was used as a model protein because it is one of the most abundant proteins in circulatory system of a wide variety of organisms and one of the most extensively studied proteins at all. It possesses a wide range of physiological functions involving the binding, transport and deposition of many endogenous and exogenous ligands present in blood circulation [5, 6]. It is well known that

[1] Department of Chemistry, Faculty of Education, University of Hradec Králové, Rokitanského 62, 500 03 Hradec Králové, Czech Republic, tel. +420 49 333 11 59, fax +420 49 333 11 66, email: lucie.trnkova@uhk.cz
[2] Department of Biochemical Sciences, Charles University in Prague, Faculty of Pharmacy, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic

many drugs are bound to serum albumin and that their effectiveness depends on their binding ability [6]. But on the other hand, drugs can cause various changes in protein conformation, which may influence its physiological function and these impaired proteins may be consequently pathologically accumulated in body tissues. Spectroscopic techniques are an ideal tool to observe conformational changes in structure of protein since it allows non-destructive measurements of compounds present at low concentration under physiological conditions. In the presented study the interactions of HCAs with the molecule of BSA were studied using UV-VIS absorption spectroscopy and fluorescence quenching method which is based on quenching of protein fluorescence by drug. The Stern-Volmer analysis is often used in order to analyze obtained data and to elucidate protein-drug (ligand) binding mechanism [7].

Experimental

Chemicals and preparation of stock solutions

Bovine serum albumin and all hydroxycinnamic acids (Fig. 1) were obtained from Sigma-Aldrich GmbH, Germany. All other chemicals were of analytical grade. Bovine serum albumin was dissolved in sodium phosphate buffer (pH 7.4; 0.1 M; 0.05% sodium azide) in order to yield solution with concentration of 2 μM for fluorescence spectroscopic experiments. Individual HCAs were dissolved in 20 mM NaOH in order to yield 10 mM stock solutions. BSA and HCA solutions were freshly prepared before each measurement.

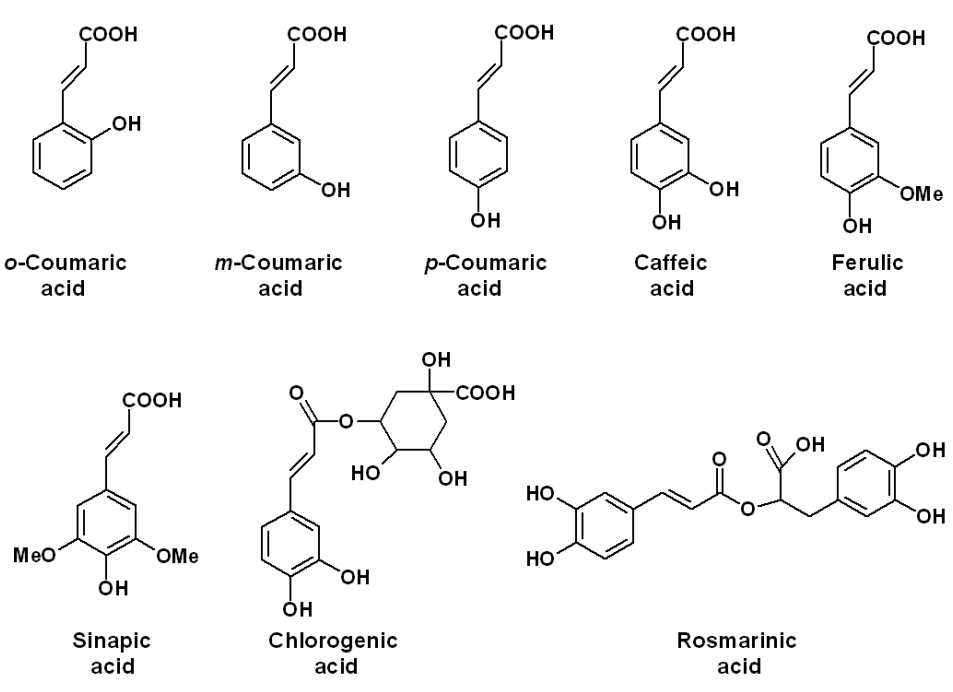

Fig. 1. Chemical structures of tested hydroxycinnamic acids

UV-VIS absorption spectroscopy

Absorption spectra were measured using spectrophotometers Lambda 25 (Perkin Elmer, United Kingdom) and Helios β (Spectronic Unicam, United Kingdom) in a 10 mm quartz cuvette. 50 μM solutions of HCAs in sodium phosphate buffer (pH 7.4; 0.1 M; 0.05% sodium azide) were incubated for up to 28 days at 37°C. The absorption UV-VIS spectra were recorded from 190 to 550 nm at the time 0, 1 hour and day 1, 7, 14, 21, and 28. The sample temperature was maintained at 37°C.

Fluorescence spectroscopy

Fluorescence spectra were recorded using a luminescence spectrometer LS 50B (Perkin Elmer, United Kingdom) in a 10 mm quartz Suprasil cuvette. Quantitative analysis of the potential interaction between individual HCA and BSA was performed by the fluorimetric titration. Briefly, solution of BSA (2 μM) was titrated in cuvette by successive additions of HCA solution (10 mM) to a final concentration of 20 μM. Fluorescence emission spectra were recorded from 300 to 530 nm with excitation at 295 nm while stirring. The excitation and emission slits were both set to 5 nm and scanning speed to 200 nm/min. All experiments were carried out at 37°C. Fluorescence intensity was read at emission wavelength of 350 nm.

Principles of fluorescence quenching

Dynamic (collisional) quenching occurs when the excited-state fluorophore is deactivated upon contact with some other molecule (quencher) and no molecule is chemically altered during this process. In the case of static quenching, a non-fluorescent complex is formed between molecules of fluorophore and quencher. Collisional quenching of fluorescence is described by the well-known Stern-Volmer equation as follows [7]

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

In this equation, F_0 and F are the fluorescence intensities of BSA in the absence and presence of quencher, respectively, [Q] is the quencher concentration, k_q is the bimolecular quenching constant, and τ_0 is the lifetime of the fluorophore in the absence of quencher. The Stern-Volmer quenching constant (K_{SV}) is given by $k_q\tau_0$. The static and dynamic quenching can be distinguished by the Stern-Volmer diagram (dependence of F_0/F on quencher concentration). When the plot of Stern-Volmer diagram shows exponential dependence, both static and dynamic quenching is present. In case the plot shows linear dependence, the quenching is either purely static or dynamic. One criterion for distinguishing of type of quenching is the fact that the obtained bimolecular quenching constant k_q is larger than the limiting diffusion rate constant of the biomolecule (2.0×10^{10} $dm^3 \cdot mol^{-1} \cdot s^{-1}$) [7], and so the static quenching process can be postulated to obtain the binding parameters according to the following equation

$$\log \frac{F_0 - F}{F} = \log K_b + n \log [Q] \tag{2}$$

where K_b represents binding constant for quencher-protein interaction, n the number of binding sites per BSA, and F_0, F have the same meaning as in Eq. (1). The values of K_b and

n could be measured from the intercept and slope by plotting log $(F_0 - F)/F$ against log [Q] [2]. Utilizing K_b, the free energy change (ΔG^0) value can be estimated from the following equation [8]

$$\Delta G^0 = -R\ T \ln K_b \tag{3}$$

Results and discussion

Changes in UV-VIS absorption spectra were employed to check stabilities of stock solutions of hydroxycinnamic acids. Each HCA has characteristic profile of its UV-VIS spectrum at physiological conditions (pH 7.40; 37°C). No changes in UV-VIS spectra of monosubstituted derivatives (coumaric acids) were observed during their incubation. Profile of spectrum of ferulic acid was not altered throughout the incubation, but the intensity of absorbance in maximum slowly decreased. The spectra of other four tested derivatives (caffeic, sinapic, chlorogenic, and rosmarinic acid) showed remarkable changes (Fig. 2) which suggest that these compounds were decomposed probably to catechol and its substituted derivatives within first 24 hours of incubation. It can be concluded that coumaric acids are stable within the course of long term incubation, while di- and trisubstituted derivatives decompose easily and therefore they are not suitable for long lasting experiments.

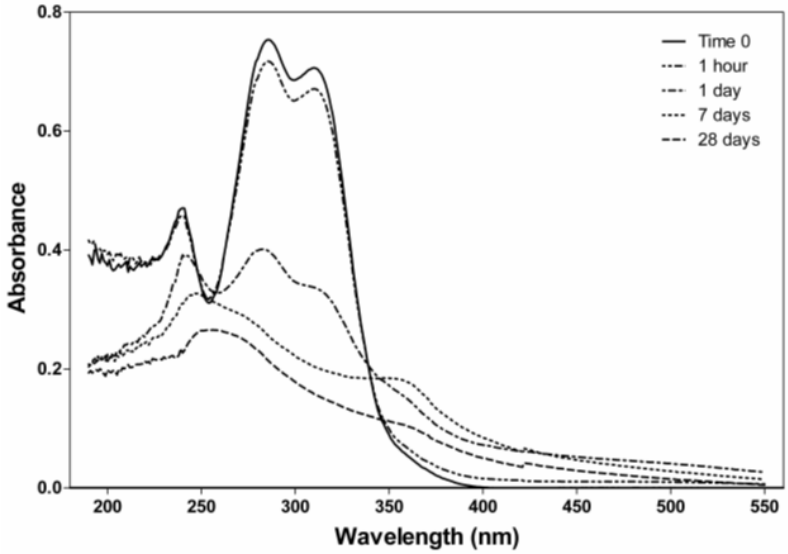

Fig. 2. Effect of long term incubation (28 days at 37°C) on characteristic UV-VIS absorption spectrum of 50 μM caffeic acid in sodium phosphate buffer (pH 7.4; 0.1 M; 0.05% sodium azide)

Quenching of protein intrinsic (tryptophan) fluorescence was employed for more detailed study of HCA-BSA binding. Fluorescence emission spectra were recorded upon excitation at 295 nm and possessed maximum at 350 nm. The excitation at 295 nm was used to ensure that the light was absorbed by tryptophan residues only [7]. Protein solution was titrated by successive additions of individual HCAs solutions. Fluorescence intensitiy

of BSA gradually decreased with increasing concentration of hydroxycinnamic acid (Fig. 3). This may indicate alterations in microenvironment around tryptophan residues in protein molecule upon interaction with tested compound. Red shifts of protein fluorescence emission bands in dependence on increasing concentration of tested compound were observed in the case of sinapic, chlorogenic and rosmarinic acids. Emission maximum of BSA was shifted by 2, 4 and 5 nm towards longer wavelength after BSA interaction with sinapic, chlorogenic, and rosmarinic acids in 20 μM concentration, respectively. Significant red shifts by 12 and 18 nm were observed by other authors upon interaction of human serum albumin with sinapic acid [9] and chlorogenic acid [10] but the concentrations of tested compounds that caused so remarkable shifts of emission maxima were as much as 150 μM. The red shift of protein emission band is caused by decrease in hydrophobic property of binding cavity near tryptophan in BSA suggesting that tryptophan has been brought to more hydrophilic environment [10] and protein secondary structure has been changed [11]. Other tested HCAs did not show any shifts in their spectra, which indicates that these HCAs can bind to BSA without affecting the immediate environment of the tryptophan residues [8].

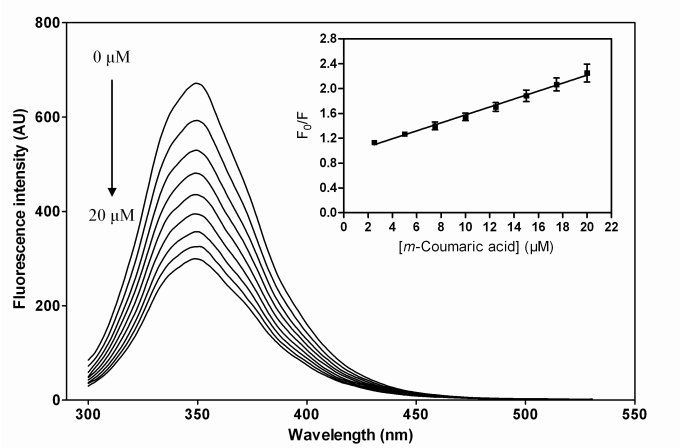

Fig. 3. Fluorescence emission spectra of BSA (2 μM) in the absence and in the presence of increasing amounts of *m*-coumaric acid (0÷20 μM) in sodium phosphate buffer (pH 7.4; 0.1 M; 0.05% sodium azide) at λ_{ex} = 295 nm and 37°C. The insert shows the corresponding Stern-Volmer diagram of the *m*-coumaric acid-BSA system (λ_{em} = 350 nm), R^2 = 0.9951

The type of fluorescence quenching was determined using the Stern-Volmer diagrams. Apart from rosmarinic acid the linear dependence in the Stern-Volmer diagram was found out in all tested HCAs and type of quenching was distinguished by the bimolecular quenching constants (k_q). In the case of rosmarinic acid the plot exhibited exponential dependence (Fig. 4) indicating that both static and dynamic type of quenching was asserted. The bimolecular quenching constants (k_q) of the rosmarinic acid-BSA system were obtained from the linear range (concentrations of 0÷10 μM) of the diagram. The bimolecular quenching constants of all HCAs (Table 1) were found to be higher than 10^{10} $dm^3 \cdot mol^{-1} \cdot s^{-1}$ [7] which is the maximum value of k_q for a diffusion controlled quenching process. This fact suggests that the static quenching mechanism between each

HCA and BSA is the main reason of protein fluorescence quenching and binding parameters can be determined according to the equation (2) (Fig. 5). The binding constants (K_b), binding sites (n) and free energy change (ΔG^0) are showed in Table 1. The binding affinity was the strongest for rosmarinic acid and ranked in the order rosmarinic acid > chlorogenic acid > sinapic acid > caffeic acid > ferulic acid > *o*-coumaric acid > *p*-coumaric acid > *m*-coumaric acid. The obtained results suggest that the binding affinity depends on the number and the position of hydroxyl groups. Di- and trisubstituted derivatives have stronger binding affinity than monosubstituted derivatives. Binding of rosmarinic acid to BSA proceeds the most easily due to higher number and suitable position of hydroxyl groups on the aromatic ring. The negative sign for ΔG^0 indicates that the binding of HCAs to the molecule of BSA is spontaneous process.

Table 1

The bimolecular quenching constants (k_q), the binding constants (K_b), the number of binding sites (n) and the free energy change (ΔG^0) of the HCA-BSA system at 37°C

Tested compound	k_q[1] [$x10^{13}$ $dm^3 \cdot mol^{-1}s^{-1}$]	K_b [$x10^5$ $dm^3 \cdot mol^{-1}$]	n	ΔG^0 [$kJ \cdot mol^{-1}$]
o-coumaric acid	1.19	3.34	1.17	–32.73
m-coumaric acid	1.19	1.31	1.08	–30.36
p-coumaric acid	1.43	1.81	1.10	–30.98
caffeic acid	0.86	4.16	1.18	–33.12
ferulic acid	0.97	3.39	1.18	–32.75
sinapic acid	0.85	4.19	1.21	–33.36
chlorogenic acid	1.07	6.67	1.23	–34.55
rosmarinic acid	16.39[2]	96.16	1.40	–41.43

Standard deviation (mean value of three independent measurements) was lower than 10%.
[1] $k_q = K_{SV}/\tau_0$; $\tau_0 = 5x10^{-9}$ s [7]
[2] assessed in the linear range (1÷10 μM)

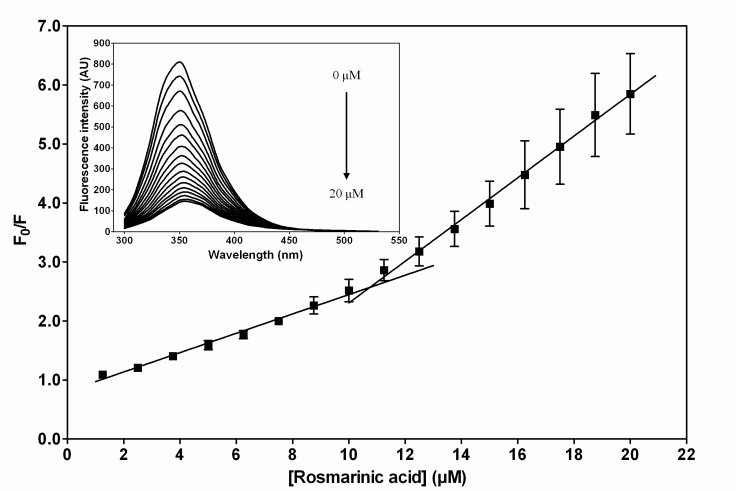

Fig. 4. The Stern-Volmer diagram of the rosmarinic acid-BSA system obtained by the titration with rosmarinic acid at physiological conditions (37°C; pH 7.4). [BSA] = 2 μM, [rosmarinic acid] = 0÷20 μM, λ_{ex} = 295 nm and λ_{em} = 350 nm. R_1^2 = 0.9895 and R_2^2 = 0.9957. The insert shows the corresponding fluorescence quenching spectra

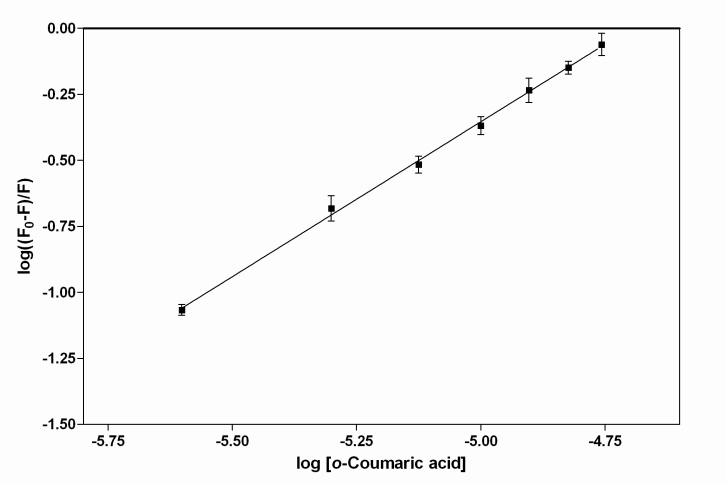

Fig. 5. Logarithmic plots of fluorescence quenching of BSA treated with different concentrations of *o*-coumaric acid at physiological conditions (37°C; pH 7.4). [BSA] = 2 μM, [*o*-coumaric acid] = 0÷20 μM, λ_{ex} = 295 nm and λ_{em} = 350 nm. R^2 = 0.9983

Conclusions

It can be concluded that coumaric acids are stable within the course of long term incubation, while di- and trisubstituted derivatives decompose easily and therefore they are not suitable for long lasting experiments. All HCAs are able to bind spontaneously to the protein molecule with different affinity depending on the number and the position of hydroxyl groups. This binding may influence physiological function of altered protein.

Acknowledgements

The presented study was supported by the Specific research 2008 of the University of Hradec Králové and by the Development project of Czech Ministry of Education No. 141/2008.

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ANTYOKSYDANTY A STRES ŚRODOWISKOWY, BADANIA SPEKTRALNE STABILNOŚCI SUBSTANCJI NATURALNYCH I ICH ODDZIAŁYWANIE Z MOLEKUŁĄ BIAŁKA

Abstrakt: Badano trwałość ośmiu kwasów hydrooksycynamonowych, HCAs, podczas inkubacji długoterminowej w warunkach fizjologicznych i możliwość ich wiązania się z modelowym białkiem (albuminą, BSA). Uzyskane wyniki pokazały, że trwałość tych kwasów zależy od ich indywidualnej budowy oraz czasu inkubacji. Wyznaczono stałe trwałości (K_b), liczbę miejsc wiązania (n) oraz zmianę wartości energii Gibbsa (ΔG^0) wiązania HCAs do BSA, który to proces przebiegał samorzutnie.

Słowa kluczowe: stres oksydacyjny, antyoksydant, kwas hydrooksycynamonowy, albumina (BSA), wiązanie białka z ligandem