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ANTIOXIDANTS AND ENVIRONMENTAL STRESS: EFFECT OF LOW-MOLECULAR COMPOUNDS ON MOLECULE OF PROTEIN

ANTYUTLENIACZE I ICH AKTYWNOŚĆ ŚRODOWISKOWA. WPŁYW ZWIĄZKÓW DROBNOMOLEKULARNYCH NA PROTEINĘ

Abstract: The aim of this study was to evaluate the relationships between the chemical structure of tea catechins and their binding affinities to HSA (*Human Serum Albumin*) by fluorescence quenching method at pH 7.4 and 37°C. The quenching constants (K_q), binding constants (K_b), and free energy changes (ΔG^0) were determined for the tested systems. The presented study contributes to the current knowledge in the area of protein-ligand binding, particularly tea catechin-HSA interactions.

Keywords: environmental oxidative stress, antioxidant, tea catechin, serum albumin, protein-ligand binding

Human organism is constantly exposed to undesirable effects of various environmental pollutants including free radicals. Although their properties vary markedly, all free radicals have one common feature - causing oxidative stress (also known as environmental stress) [1]. Oxidative stress plays an important role in the pathogenesis of many human diseases and in the physiological process of aging [2]. Therefore, considerable attention has been focused on the study of naturally occurring substances with antioxidant activity, which could protect human organism against environmental pollutants [3]. Tea catechins are ranged among these outstanding compounds and exert a broad spectrum of biological activities including antioxidant properties [4]. Serum albumin is one of the most abundant proteins in circulatory system and possesses a wide range of physiological functions involving the binding, transport and deposition of many endogenous and exogenous ligands.

It is well known that many low-molecular drugs are bound to serum albumin and their effectiveness thus depends on their binding ability [5]. On the other hand, drugs can cause various changes in protein conformation which may influence its physiological function. Such impaired proteins may be consequently accumulated in body tissues. Spectroscopic techniques including fluorescence spectroscopy represent an ideal tool for studying conformational changes in protein structure since they allow non-destructive measurements of compounds present at low concentration under various conditions [6]. The aim of this study was to evaluate the relationships between the chemical structure of tea catechins and their binding affinities to HSA (*Human Serum Albumin*) in detail using fluorescence quenching method at pH 7.4 and 37°C.

Materials and methods

Human serum albumin and tea catechins (Fig. 1) were obtained from Sigma-Aldrich GmbH, Germany. All other chemicals were of analytical grade.

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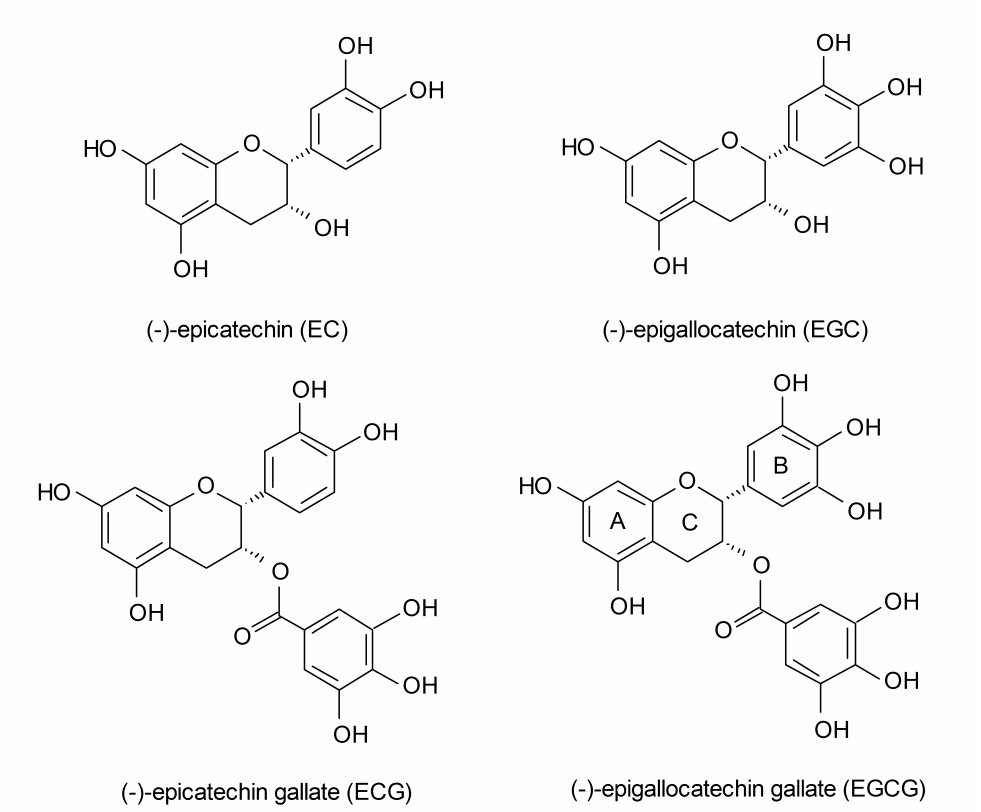

Fig. 1. Structures of tested catechins

Human 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 4 x 10^{-6} M. Catechins were dissolved in anhydrous methanol in order to yield 1 x 10^{-2} M stock solutions.

Fluorescence spectra were recorded using a luminescence spectrometer LS 50B (Perkin Elmer) with a 10 mm quartz Suprasil cuvette. Quantitative analysis of the potential interaction between catechins and HSA was performed by the fluorimetric titration. Briefly, solution of HSA (4 x 10^{-6} M) was titrated in cuvette by successive additions of catechin solution (1 x 10^{-2} M) to a final concentration of 4 x 10^{-5} M. Fluorescence emission spectra were recorded from 300 to 530 nm with excitation at λ = 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. Appropriate blanks were subtracted to correct the fluorescence background. Fluorescence intensity was read at emission wavelength of 348 nm which corresponds with the emission maximum of HSA.

All measurements were performed three times. The mean values of constants and standard deviations were calculated. Standard deviations were always lower than 10%.

Results and discussion

Fluorescence quenching mechanism

Quenching of protein intrinsic (tryptophan) fluorescence was employed for more detailed study of tea catechin-HSA interactions. Fluorescence intensity of HSA gradually decreased with increasing concentration of all tested catechins (Fig. 2). Tea catechins with the galloyl group on the C-ring (ECG and EGCG) caused more pronounced decrease in the tryptophan fluorescence than the non-galloylated catechins (EC and EGC). Moreover, the galloylated catechins induced red shift in the protein emission maximum (shift to longer wavelength about 20 and 25 nm for EGCG-HSA and ECG-HSA system, respectively). The red shift is caused by decrease in a hydrophobic property of binding cavity near sole tryptophan (Trp-214) in HSA molecule suggesting that this tryptophan residue has been brought to a more hydrophilic environment and protein secondary structure has been changed [7]. Significant red shift in EGCG-HSA system was observed also by Maiti et al [8]. No shift in the emission maximum of HSA was observed in the case of the non-galloylated catechins.

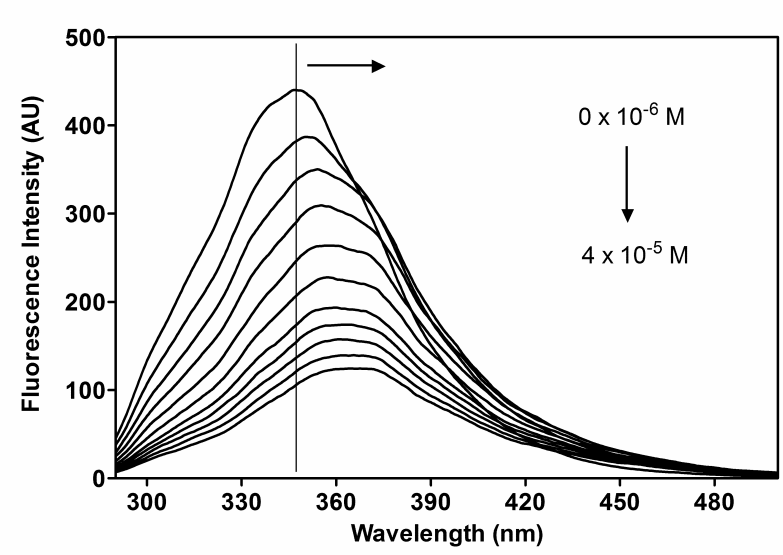

Fig. 2. Fluorescence quenching spectra of HSA (4 x 10^{-6} M) in the increasing concentration of epigallocatechin gallate (0-4 x 10^{-5} M) in sodium phosphate buffer (pH 7.4, 0.1 M, 0.05% sodium azide) at λ_{ex} = 295 m and 37°C

In order to clarify the fluorescence quenching mechanism induced by the tested catechins, the fluorescence quenching data were analyzed using the Stern-Volmer equation:

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

where F_0 and F are the fluorescence intensities of HSA in the absence and presence of the quencher, respectively. [Q] is the quencher concentration, K_{SV} is the Stern-Volmer

constant, K_q is the bimolecular quenching rate constant and τ_0 is the lifetime of the fluorophore in the absence of the quencher (τ_0 is about 5 ns) [9].

The Stern-Volmer constants (K_{SV}) and bimolecular quenching rate constants (K_q) were determined from the eq. (1) using linear regression of the plot of F_0/F versus [Q] (Table 1). The representative Stern-Volmer plot of EC-HSA system is displayed in Figure 3. All values of K_q were much greater than the diffusion-limited rate constant of the biomolecule (K_{diff} = 1.0 x 10^{10} l $mol^{-1}s^{-1}$) which suggested that the static quenching mechanism is dominant in the studied interactions [9]. The highest value of K_q was determined for epicatechin gallate and further decreased in the order ECG > EGCG >> EC > EGC. The importance of the galloyl moiety on the C-ring was evident because quenching constants for the catechin gallates (ECG and EGCG) were significantly higher than those of catechins lacking the galloyl group (EC and EGC). Catechins with the catechol group on the B-ring (EC and ECG) showed more pronounced quenching effect than their analogs with the pyragollol group (EGC and EGCG).

Table 1

The Stern-Volmer constants (K_{SV}), bimolecular quenching rate constants (K_q), binding constants (K_b), and free energy changes (ΔG^0) of the catechin-HSA systems (pH 7.4, 37°C)

Tested compound	K_{SV} [10^4 M^{-1}]	K_q [10^{12} $M^{-1}s^{-1}$]	K_b [10^4 M^{-1}]	ΔG^0 [kJ·mol^{-1}]
Epicatechin	1.19	2.38	2.58	–26.18
Epigallocatechin	0.46	0.93	1.92	–25.42
Epicatechin gallate	11.62	23.24	6.86	–28.70
Epigallocatechin gallate	9.19	18.39	6.80	–28.68

$K_q = K_{SV}/\tau_0$; τ_0 = 5 x 10^{-9} s [9]. All correlation coefficients (R) were higher than 0.990. Standard deviations (mean value of three independent experiments) were lower than 10%.

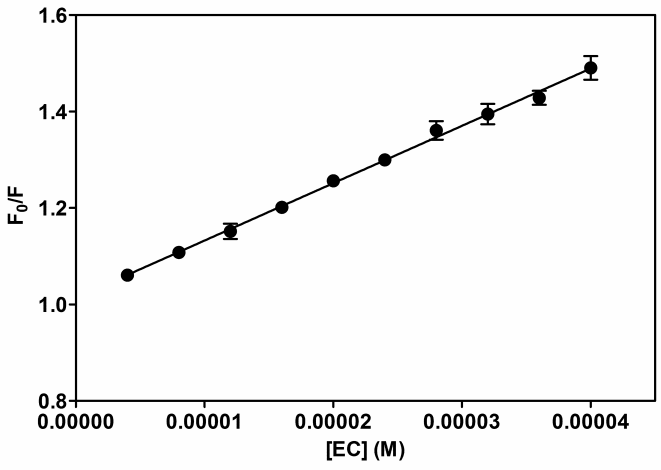

Fig. 3. The Stern-Volmer plots of epicatechin-HSA system at pH 7.4 and 37°C. [HSA] = 4 x 10^{-6} M, [EC] = 0-4 x 10^{-5} M, λ_{ex} = 295 nm, λ_{em} = 348 nm, y = 11890x + 1.014, and R = 0.995

Binding parameters

The binding constants (K_b) of catechin-HSA systems were calculated using the following Lineweaver-Burk equation [10]:

$$1/(F_0 - F) = 1/F_0 + 1/(K_b F_0[Q]) \quad (2)$$

where F_0 and F are the fluorescence intensities before and after the addition of the quencher, [Q] is the quencher concentration. The free energy change (ΔG^0) value was estimated from the following equation [11]:

$$\Delta G^0 = -RT\ln K_b \quad (3)$$

where R is the gas constant (8.314 J K^{-1} mol^{-1}) and T is temperature (T = 310 K).

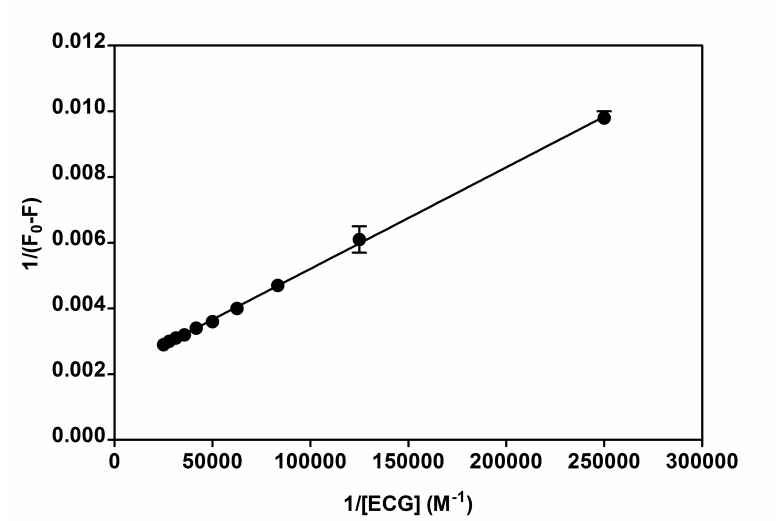

Fig. 4. The Lineweaver-Burk plots for epicatechin gallate-HSA system at pH 7.4 and 37°C. [HSA] = 4 x 10^{-6} M, [ECG] = 0-4 x 10^{-5} M, λ_{ex} = 295 nm, λ_{em} = 348 nm, y = 3.09 x 10^{-8}x + 2.23 x 10^{-3}, and R = 0.998

The binding constants (K_b) were obtained by plotting of $1/(F_0 - F)$ versus 1/[Q] (Fig. 4). The binding affinity was the strongest for epicatechin gallate and decreased in the order ECG > EGCG >> EC > EGC. The free energy changes were calculated using the eq. (3). The obtained results are summarized in the Table 1. The galloylated catechins (ECG and EGCG) showed significantly higher binding ability than the non-galloylated catechins (EC and EGC) due to the presence of the galloyl group on the C-ring, ie the additional aromatic ring and three hydroxyl groups which can establish hydrophobic interactions and hydrogen bonds, respectively. Our results are consistent with earlier obtained data by high-performance affinity chromatography with immobilized albumin column [12]. In addition, catechol-type catechins (EC and ECG) possessed stronger binding affinity than pyragollol-type catechins (ECG and EGCG) which suggests that the insertion of an additional hydroxyl group on the B-ring does not contribute to their binding affinities as was described in literature [12]. One of the most studied tea catechin is epigallocatechin gallate and the results obtained for the EGCG-HSA system by Maiti et al [8] are in good

agreement with our data although the experimental conditions were slightly different. The spontaneity of HSA-catechin interactions was confirmed by the negative values of ΔG^0.

Conclusions

The interactions of tea catechins and HSA were investigated by fluorescence quenching method. Tea catechins quenched tryptophan fluorescence of HSA mainly by static quenching mechanism in the studied range of concentrations (0-40 x 10^{-6} M). Thus, the non-fluorescent complexes were formed. All tested catechins bound spontaneously to the molecule of HSA with different binding affinity. The binding constant was the strongest for epicatechin gallate and decreased in the order ECG > EGCG >> EC > EGC. The most important structural feature of the tested catechins contributing to HSA binding was the galloyl group on the C-ring. The presented study contributes to the current knowledge in the area of protein-ligand binding, particularly tea catechin-HSA interactions.

Acknowledgements

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ANTYUTLENIACZE I ICH AKTYWNOŚĆ ŚRODOWISKOWA. WPŁYW ZWIĄZKÓW DROBNOMOLEKULARNYCH NA PROTEINĘ

Abstrakt: Celem pracy była ocena zależności między strukturą chemiczną katechin herbaty i ich powinowactwem do HSA (*albumina ludzkiej surowicy*) za pomocą metody wygaszania fluorescencji przy pH 7,4 i w temperaturze 37°C. Dla badanych układów wyznaczono stałą szybkości wygaszania (K_q), stałą wiązania (K_b) i zmiany entalpii swobodnej (ΔG^0). Prezentowana praca poszerza aktualny stan wiedzy na temat wiązania białko - ligand, zwłaszcza interakcji katechiny - HSA.

Słowa kluczowe: środowiskowy stres oksydacyjny, antyutleniacze, katechiny herbaty, albuminy surowicy, wiązanie białko - ligand