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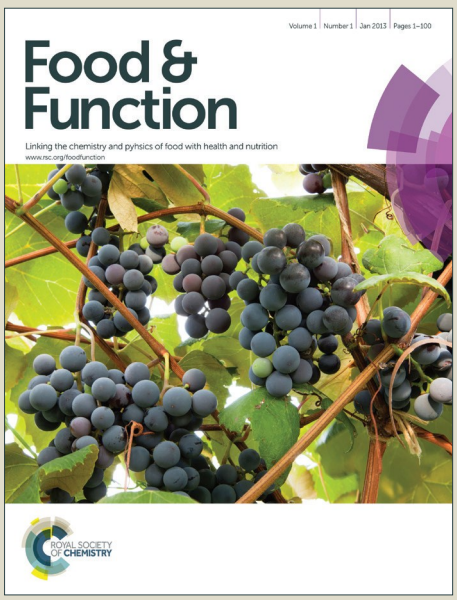

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Binding of enterolactone and enterodiol to human serum albumin: Increase of Cystein-34 thiol group reactivity

Marija M. Takić [a], Vesna B. Jovanović[b], Ivan D. Pavićević[b], Tamara N. Uzelac[b], Jelena M. Aćimović[b], Danijela K. Ristić-Medić[a], Ljuba M. Mandić [b,*]

[a]Institute for Medical Research, Center of research excellence in nutrition and metabolism, University of Belgrade, Belgrade, Serbia

[b] Department of Biochemistry, Faculty of Chemistry, University of Belgrade, Studentski trg 12-16, Belgrade 11158, Serbia

[*]Author for correspondence: Prof. Dr Ljuba Mandić

Department of Biochemistry, Faculty of Chemistry, University of Belgrade

P.O.Box 51, Studentski trg 16, 11158 Belgrade, Serbia

Tel: +381 11 333 66 76

e-mail: ljmandic@chem.bg.ac.rs

Abstract

Interaction of polyphenolic molecules with human serum albumin (HSA) could lead to changes of HSA Cys34 thiol group (HSA-SH) reactivity. The influences of enterolactone (EL) and enterodiol (ED) binding on HSA-SH reactivity in fatty acid (FA) free HSA and HSA with bound stearic acid (S) in S/HSA molar ratios (1:1 and 4:1), were investigated by determination of pseudo first order rate constant (k') for thiol reaction with 5,5'-dithiobis-(2-nitrobenzoic acid). The binding affinities and binding sites of EL and ED, using fluorescence measurement of the intrinsic fluorescence of Trp214 and diazepam (binding site marker), were determined too. EL and ED binding to HSA increases the reactivity of HSA-SH in all assayed HSA-enterolignans complexes from 9.1-33.1%. The strongest effects were obtained for FA-free HSA-enterolignans complexes. S modulates/reduces the effect of EL on HSA-SH reactivity, while its influence on the effect of ED is negligible. Investigated enterolignans binding to HSA: the binding constants were the highest for FA-free HSA (EL: 11.64 x 10^4 M^{-1} and ED: 5.59 x 10^4 M^{-1} at 37^o C) and the lowest for S/HSA 4:1-enterolignan complexes (EL: 2.43 x 10^4 M^{-1} and ED: 1.92 x 10^4 M^{-1}). With increase of S/HSA ratio binding affinities and number of binding sites for EL and ED were decreased. At the same time, high correlation between binding constants and increases of Cys34 reactivity was found (r=0.974). Competitive experiments using diazepam indicate that binding of ED and of EL was located in the hydrophobic pocket of site II in HSA. Overall, it is evident that stearic acid could modulate enterolignans effects on HSA-SH reactivity as well as binding to HSA. This finding could be important for pharmacokinetics and expression of enterolignans antioxidant effect *in vivo* after intake of lignans rich food.

Keywords: human serum albumin, thiol group reactivity, enterolactone, enterodiol, and fatty acids binding

Abbreviations: DTNB, 5, 5'-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; ED, enterodiol; EL, enterolactone; FA, fatty acid; FA-free HSA, defatted HSA; FA-bound HSA, complex HSA with FA; HSA, human serum albumin; HSA-EL and HSA-ED, HSA complexes with enterolactone and enterodiol; HSA-SH, Cys34 free thiol group of the HSA; S, stearic acid, S/HSA, complex of HSA with stearic acid.

INTRODUCTION

Human serum albumin (HSA) is the most abundant protein in plasma, present at high concentration about 0.6 mM [1], and with half-life of 19 days. As the α-helix and random structure contents in HSA are 70 % and 30 % (with almost no β-sheets), HSA molecule has high degree of conformational flexibility [2]. Crystallographic data show that HSA contains three homologous 10 helices domains (I, II and III) that can be divided in two subdomains (A and B) displaying also partial iner-subdomain homology [3]. HSA is a transporter of many endogenous substances (non-esterified FA, bilirubin, bile salts, steroid hormones, hematin, tryptophan, thyroxin and some vitamins), several metal ions and different exogenous molecules (e.g. polyphenols and drugs), that greatly augments the transport capacity of blood plasma [4]. For medium and long-chain fatty acid (FA) HSA has seven binding sites (of varying affinities) distributed throughout the molecule, which involves all six subdomains [5]. There are also four thyroxin binding sites, several metal binding sites including albumin's N-terminus, and a site centered around residue Cys34 [6]. HSA exogenous ligands are accommodated primarily to one of two major sites with binding associations' constants in a range from 10^4 to 10^6 M [2]. Drug binding site I is located in subdomain IIA (and overlaps with FA binding site 7), and drug site II in subdomain IIIA (overlaps with FA binding sites 3 and 4) [7].

Dietary phenolic substances have received much attention as numerous studies have revealed their various protective effects *in vitro* and *in vivo* [8, 9]. Enterolactone (EL) and enterodiol (ED) (Figure 1) are produced from several dietary plant lignans (with polyphenolic structure) by extensive metabolism of the gut microflora [10]. They are especially abundant in seeds, whole grains and berries [11]. Results of our previous study [12] indicated that dietary milled sesame/pumpkin/flax seeds mixture, reach in polyunsaturated FA and lignans, added to habitual diet lowered triglyceride and inflammatory markers levels, affect glycemic control, improved FA profile and pruritus symptoms in hemodialysis patients. Because of their antioxidant and weak estrogenic effect enterolignans may have many beneficial effects on human liver function [13], on decrease of risk for breast [14], prostate cancers [15] and cardiovascular diseases [16]. The variations in enterolignans serum and urine levels were high and the most of the differences within population could be attributed to different dietary habits. Beside dietary intake, metabolism by intestinal bacteria, endogenous hormones, and antibiotic use also influence lignans levels. EL and ED are mainly present as glucuronide and sulfate conjugates in body fluids and are eliminated slowly via urine [17]. The binding of absorbed phytoestrogens to albumin is an important factor determining their pharmacokinetics, pharmacodynamics and biological activities. Numerous studies reported up to now have dealt with native polyphenols binding to albumin and noticeable differences in their binding behavior, due to subtle differences in structure, were revealed [18]. To our best knowledge, interactions between enterolignans and albumin were not studied yet.

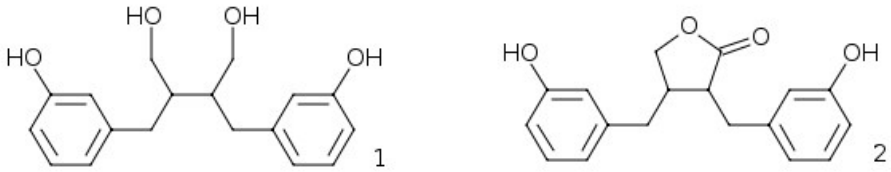

Figure 1. Structures of enterolignans: enterodiol (1) and enterolactone (2)

HSA has 17 disulfide bridges and one free Cys34 thiol group (HSA-SH) [8]. HSA-SH comprises approximately 80 % of the total free thiols in plasma [19]. About 70 % of circulating HSA contains Cys34 in reduced thiol state. The rest of Cys34 residue consists mostly of mixed disulfides with cysteine and other low molecular weight thiols [20]. A minor fraction is oxidized to higher oxidation states such as sulfinic (HSA-SO_2H) and sulfonic acid (HSA-SO_3H) [21]. Therefore, HSA has recognized as a very important antioxidant in plasma. Under normal physiological conditions, between 0.1 and 2 mol of free FA are bound to HSA, but the molar ratio of FA/HSA can rise above 6:1 in fasting, intensive exercise or under pathological conditions such as diabetes, liver and cardiovascular diseases [22]. Binding of FA is associated with significant structural changes in the HSA molecule [23], which could cause Cys34 residue to be more or less exposed to the surrounding environment, leading to differential reactivity and susceptibility to oxidative stress [19-21, 24-27]. These findings lead to the question: Does the binding of EL and ED (for which the antioxidant effect is approved) to HSA could influence on HSA-SH reactivity and therefore its antioxidant potential?

Although, numerous studies on the interactions between polyphenols and HSA have been performed, the changes of HSA-SH reactivity that can occur upon polyphenols binding (possible way of expressing their antioxidant properties) are not considered yet. Therefore, in this study the pseudo first order rate constants for HSA-SH reaction with 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB) of HSA-SH in the presence of EL and ED were determined. As the HSA binding sites for FA and polyphenols are overlapping, cooperative and competitive interactions between FA and enterolignans on the HSA-SH reactivity were also investigated. Two stearic acid/HSA (S/HSA) molar ratios (1:1 and 4:1) that correspond to normal and pathological conditions were used. In order to better understand obtained results, the interactions of EL and ED with HSA were investigated. The binding affinities and binding sites of EL and ED, using fluorescence measurement of the intrinsic fluorescence of Trp214 and diazepam (binding site marker), were determined.

MATERIALS AND METHODS

2.1. Chemicals

All chemicals were purchased from Sigma (Steinheim, Germany) unless otherwise noted. The 20 % solution of HSA was purchased from Baxter (Vienna, Austria). All chemicals used were analytical grade.

2.2. Preparation of FA-free HSA and reduced HSA

Commercial HSA contains bound free FA and contains approximately 40 % of HSA-SH in the reduced form. For experimental purposes HSA was defatted according to Chen's charcoal treatment method [27], and then reduced with dithiothreitol (DTT) as described by Penezic et al. [28]. In all experiments, HSA with free thiol group content of about 60-70 % (FA-free HSA) was prepared by mixing appropriate volumes of defatted HSA and reduced defatted HSA.

2.3. Preparation of FA-bound HSA samples, HSA-EL and HSA-ED complexes

The solution of S (50 mM) in 99 % ethanol was mixed with the solution of FA-free HSA (0.25 mM in 0.1 M sodium phosphate buffer, pH 7.4) at molar ratios 1:1 (S/HSA 1:1) and 4:1 (S/HSA 4:1). The mixtures were incubated at room temperature over night and then centrifuged (12000 rpm, 5 min). The highest increase of thiol group reactivity was observed when polyunsaturated FA (PUFA) are bound to HSA [25] (and it is known that phythoestrogens influence PUFA oxidative metabolism [29]), but they were avoided because of their oxidizability.

HSA enterolignan complexes (HSA-EL and HSA-ED) were prepared by mixing FA-free HSA, S/HSA 1:1 or S/HSA 4:1 with appropriate volume of EL or ED solutions (25 mM in DMSO) to get final HSA/enterolignan molar ratio 1:1 and incubating at 37°C during one hour.

2.4. Quantification of HSA and HSA-SH group content

Biuret assay was used for quantification of total protein content [30]. Free HSA-SH content was determined spectrophotometrically according to a modified Ellman's method [31]. All reagents were kept at room temperature 30 min before determination. DTNB reagent, 100 μL of 2 mM solution was mixed with equal volumes of sample and 1 M Tris buffer (pH 8.0) and brought up to 1000 μL with water. Absorbance was measured after 30 min at room temperature at 412 nm against the sample and reagent blanks. The concentration of thiols was calculated by using the molar extinction coefficient (14150 M^{-1} cm^{-1}) [32].

2.5. Determination of the pseudo first order constant for the reaction of HSA-SH with DTNB

Reaction kinetics was monitored spectrophotometrically using method that is described in details elsewhere [25]. Briefly, sample (100 μL) of FA-free HSA or S/HSA complexes (with EL or ED) (0.25 mM HSA, with HSA-SH content of about 60-70%) was mixed with 1M Tris buffer pH 8.0 (100 μL), water (700 μL) and 3.5 mM DTNB reagent (100 μL). The concentration of DTNB reagent represents a twenty fold pseudo first order excess compared to HSA-SH concentration. After mixing, absorbance at 412 nm was recorded every 5 s for first 90 s and then every 10 s for 270 s, then 30 s until 30 min of total reaction time. Values of k' were determined by fitting linear least squares model natural logarithm of unreacted thiol group concentrations versus time.

2.6. Fluorescence measurements

Binding of EL and ED to HSA was studied by fluorescence quenching titration method using the intrinsic fluorescence of HSA as probe. The fluorescence measurements were performed on a FluoroMax-4 Jobin Yvon (Horiba Scientific, Japan) spectrofluorometer equipped with 1.0 cm quartz cell and thermostat bath. The excitation and emission slit widths were set at 5.0 nm.

Solutions of FA-free HSA, S/HSA 1:1, S/HSA 4:1 were prepared daily by diluting of stock solution of HSA (0.25 mM in 100 mM sodium phosphate buffer pH 7.4) and enterolignans (25 mM in DMSO) with 100 mM sodium phosphate buffer pH 7.4 to final concentration of HSA (0.5 μM) and enterolignans (100 μM) in all experiments. Small aliquots of 100 μM ligands solutions were added to 2.5 ml of 0.5 μM HSA solution. So the final concentrations of ligands were: 0.125, 0.25, 0.5, 1.0, 1.5, 2.0 and 2.5 μM. Excitation wavelength was 280 nm and emission spectras in range between 300 to 500 nm were recorded at 37°C.

Ligands (quencher) can absorb energy both at the HSA excitation and emission wavelengths. In order to overcome the inner-filter effect, the absorbance values of used ligands were recorded on a Shimadzu UV 1800 (Japan) and corresponding corrections were made during calculation of binding parameters according to formula 1 [33]:

$$F_c = F_u \times 10^{(A_{ex} \times d_{ex} + A_{em} \times d_{em})/2} \quad (1)$$

where F_u is measured emission fluorescence intensity, F_c is corrected fluorescence intensity that would be measured in absence of inner–filter effect, d_{ex} and d_{em} are the cell path lengths in the excitation and emission direction (1cm), A_{ex} and A_{em} are absorbance values of quencher measured at the excitation and pick emission wavelength (340nm).

The quenching constants of HSA/enterolignans complexes were determined using Stern-Volmer's equation 2:

$$F_0/F = 1 + k_q \tau_0 [Q] = 1 + K_{sv} [Q] \quad (2)$$

where F_0 and F are the HSA fluorescence intensities at 340 nm before and after addition of the quencher (EL or ED), K_{sv} is the Stern-Volmer's quenching constant, k_q stands for the fluorescence quenching rate constant, τ_0 is the fluorescence lifetime of fluorophore and [Q] is quencher concentration [34].

The estimation of association (binding) constants (K_a) and number of binding sites (n) of HSA and enterolignans (EL and ED) was done using equation 3 [35]:

$$\log (F_0\text{-}F)/F = -n \log (1/ ([Q]-[P]x(F_0\text{-}F)/F_0) + n \log K_a \quad (3)$$

where [Q] and [P] are total concentration of ligands (EL, ED) and proteins (FA-free HSA, S/HSA 1:1 and 4:1).

In the binding site marker experiments, for site II diazepam was used as marker. The 2.5 ml of 0.5 μM HSA-EL or HSA-ED were titrated with 100 μM diazepam (daily prepared by diluting stock solution of 50 mM in DMSO with 100 mM sodium phosphate buffer, pH 7.4) and final concentration of diazepam varied from 0-1.5 μM at an increment of 0.125 μM.

2.7. Statistical analysis and graphing

All statistical analysis and graphical represents of data were performed using Origin 9.0 statistical program.

3. RESULTS AND DISCUSSION

The cooperative and competitive interactions between FA and polyphenolic molecules (e.g. enterolignans) could be important factor effecting HSA-SH reactivity. Therefore, the reactivity of FA-free HSA-SH and FA/HSA-SH complexed with enterolignans, that are appropriate physiological models: S/HSA 1:1 (normal physiological condition) and S/HSA 4:1 (in fasting, intensive exercise or under pathological conditions), was first investigated.

3.1. Influence of stearic acid on the reactivity HSA-SH

The reactivity of HSA-SH was studied with low molecular weight disulfide DTNB reagent, at the concentration twenty times fold compared to HSA-SH that represented pseudo-first order excess. The reaction between HSA Cys34 thiol group and DTNB can be written as follows:

$$HSA\text{-}S^- + DTNB \rightarrow HSA\text{-}S\text{-}TNB + TNB^- \quad (4)$$

Time courses (0–30 min) of the reactions of HSA-SH were monitored spectrophotometrically. Graphics obtained after linearization of kinetics data show that reactions followed pseudo-first order reaction kinetic (Figure 2). The values of k' obtained for FA-free HSA and S/HSA 1:1 and 4:1 were $8.9 \pm 0.1 \times 10^{-3}$ s^{-1}, $11.6 \pm 0.3 \times 10^{-3}$ s^{-1} and $15.3 \pm 0.1 \times 10^{-3}$ s^{-1}, respectively. They are in accordance with previous results obtained at similar conditions [25]. The reactivity of HSA-SH of S/HSA 4:1 complex was almost two times higher than of FA-free HSA. The albumin free thiol group presented at a concentration of 0.6 mM in plasma constitutes the largest pool of reactive thiols in plasma and stands as key oxidant scavenger [19-21]. Therefore, in some physiological conditions (e.g. exercise, fasting) and diseases (hemodialysis, preeclampsia, diabetes) which are associated with oxidative stress, tend to increase the amount FA bound to HSA [19] could be a protective adaptation. Reactivity of HSA-SH is increased upon FA binding and depended on type of FA bound to HSA [25]. It is reported that upon FA binding, the environment of the HSA-SH could become more polar and induced conformation changes may cause increased accessibility of Cys34 group [20].

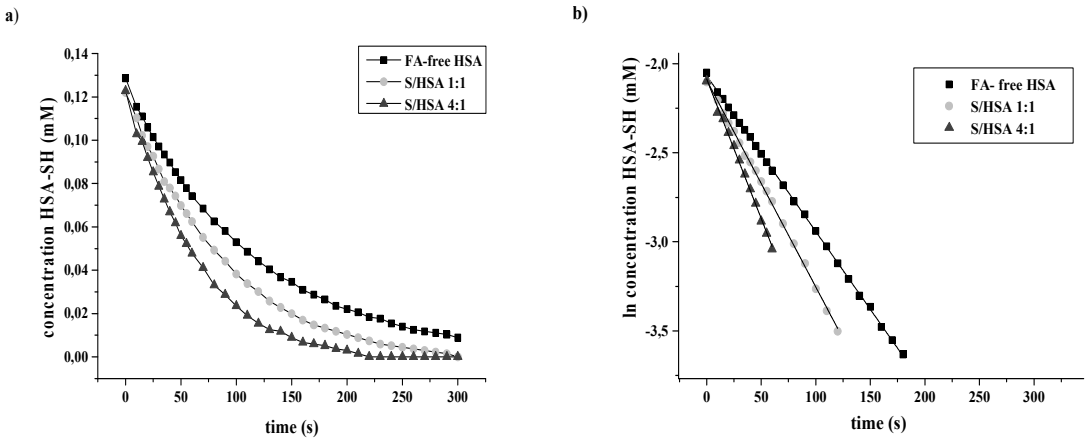

Figure 2. (a) Time courses curves obtained for the reaction of HSA-SH of FA-free HSA, S/HSA 1:1 and 4:1 with DTNB. (b) Linear models of pseudo first order reaction kinetics of FA-free HSA, S/HSA 1:1 and 4:1 thiol group.

3.2. Binding enterolignans to HSA influences Cys34 thiol group reactivity

The influence of enterolignans (ED and EL) binding on the reactivity HSA-SH was investigated after incubation of FA-free HSA or S/HSA 1:1 or 4:1 (0.25 mM) at 37°C for one hour with EL and ED in molar ratio 1:1.

Significant increases in the reactivity of HSA-SH after binding of enterolignans to all HSA complexes without and with FA, were found (Figure 3, Table 1). The highest effect on HSA-SH reactivity was obtained for FA-free HSA-EL followed with FA-free HSA-ED (33.1 and 13.6 %, respectively). Even though, the thiol group of formed complex FA-free HSA-EL had reactivity similar to S/HSA 1:1 (k' values $11.8 \pm 0.4 \times 10^{-3}$ s^{-1} vs. $11.6 \pm 0.3 \times 10^{-3}$ s^{-1}, Table 1). Thus, the values obtained for k' constants lead to conclusion that enterolignans binding to HSA lead to the increase of HSA-SH reactivity and this effect was more pronounced at lower molar ratios FA/HSA.

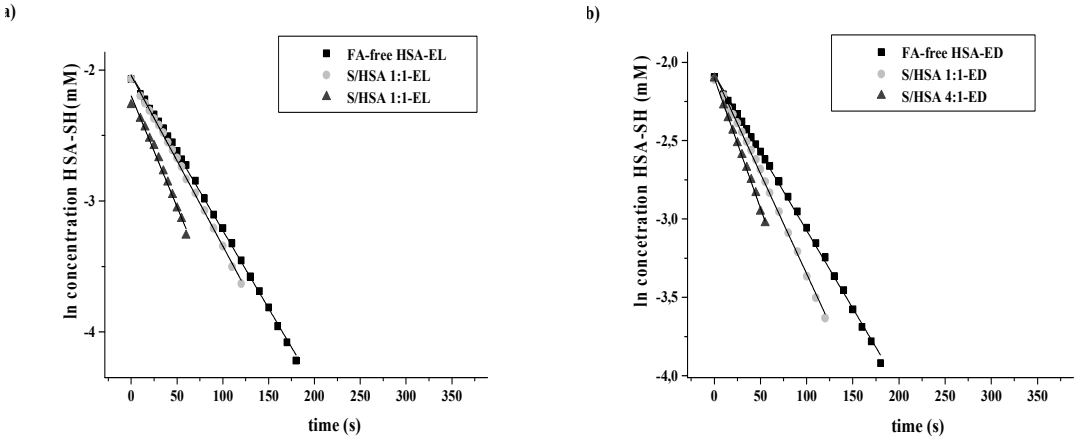

Figure 3. Linear models of pseudo first order reaction kinetics of FA-free HSA, S/HSA 1:1 and 4:1 thiol group with DTNB, obtained after binding of (a) enterolactone and (b) enterodiol.

Table 1. Pseudo first order rate constants (k´) for the reaction of thiol group of FA-free HSA, S/HSA (1:1 and 4:1), and their complexes with enterolignans. Each experiment is done in triplicate.

Complex	k´ x 10^{-3} s^{-1}	Increase (%) of HSA-SH reactivity after binding of	
		Enterolignans[a]	S + enterolignans[b]
FA-free HSA	8.9 ± 0.1		
FA-free HSA-EL	11.8 ± 0.4	33.1	
FA-free HSA-ED	10.1 ± 0.1	13.6	
S/HSA (1:1)	11.6 ± 0.3		
S/HSA (1:1) -EL	13.0 ± 0.6	12.6	46.7
S/HSA (1:1) -ED	12.8 ± 0.3	10.9	44.4
S/HSA (4:1)	15.3 ± 0.1		
S/HSA (4:1) -EL	17.0 ± 0.3	10.8	91.1
S/HSA (4:1) -ED	16.7 ± 0.9	9.1	88.1

[a] compared to FA-free HSA, S/HSA (1:1) and (4:1), resp.; [b] compared to FA-free HSA

In S/HSA complexes 1:1 and 4:1 reactivity of Cys34 was increased by 30.3 % and 72.4 % compared with FA-free HSA, respectively. Overall, it is evident that both enterolignans (EL and ED) and S interactions with HSA increased the reactivity of HSA-SH in all investigated HSA complexes (Table 1). Singular contributions of S and enterolignans (ED, EL) to increase of HSA-SH reactivity were summed and compared with cumulative increases of HSA-SH reactivity (Figure 4). As it can be seen from Table 1 EL led to the increase of FA-free HSA-SH reactivity by 33.1 %. If this value was summed with 30.3 % increase of HSA-SH reactivity contributed by S interactions with FA-free HSA, the sum of singular effects of EL and S would be 63.4 % (Figure 4), but the obtained increase of HSA-SH reactivity in S/HSA (1:1)-EL complex was 46.7 % (Table 1, Figure 4). Thus, it is evident that S modulated/reduced the effect of EL on reactivity of HSA-SH group and this influence was in small extent more pronounced at lower molar ratios FA/HSA (decrease of 16.7 % in comparison to 14.4 %, resp.). At the same time, the negative effect of S on ED increase of HSA-SH reactivity was not found (Figure 4). The finding that the reactivity of HSA-SH was increased in HSA-enterolignans complexes could be important for possible modulation of HSA-SH reactivity by dietary intake of lignans rich food. In some physiological conditions and diseases with elevated plasma levels of non-esterified FA the effects of dietary intake of lignans rich food could be modulated by FA.

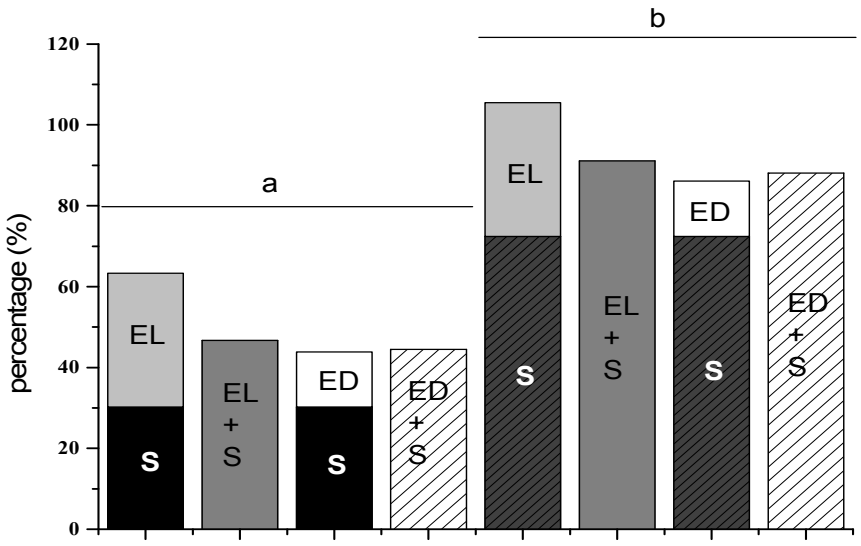

Figure 4. Singular and cumulative contribution of stearic acid (S) and enterolignans (EL and ED) to increase of reactivity of Cys34 group of S/HSA 1:1 (a) and S/HSA 4:1 (b) compared to reactivity of FA-free HSA

3.3. Effect of enterolactone and enterodiol binding to HSA on intrinsic fluorescence

In order to better understand influences of enterolignans on HSA-SH reactivity, the interactions of EL and ED with HSA and FA/HSA were investigated using fluorescence spectroscopy. The fluorescence excitation wavelength was 280 nm, at which both Trp214 and Tyr residues emit fluorescence. In spite of the presence of 18 Tyr residues in HSA molecule [36] the HSA intrinsic fluorescence is dominated by single Trp214 in subdomain IIA [34].

Due to binding of EL or ED to FA-free HSA and S/HSA 1:1 and 4:1 molecule (results for enterolignans/HSA molar 0.5:1 and 3:1 are shown in Figure 5a, 5b and 5c), the quenching of fluorescence intensity at λ_{em} (340 nm) compared to the FA-free HSA and S/HSA 1:1 and 1:4 was observed. In the presence of EL and ED the intrinsic fluorescence decreased in a concentration-dependent manner, suggesting that EL and ED interact with HSA. The blue shift of emission maximum wavelength (FA-free HSA-EL and S/HSA 4:1-EL) was occurred, suggesting that the intrinsic fluorophore could be forced in more hydrophobic protein environment [37] in the presence of high concentrations of EL. Observed more pronounced HSA intrinsic fluorescence changes (i.e. conformational changes) induced by EL binding in comparison to ED are in accordance to the their effects on HSA-SH reactivity.

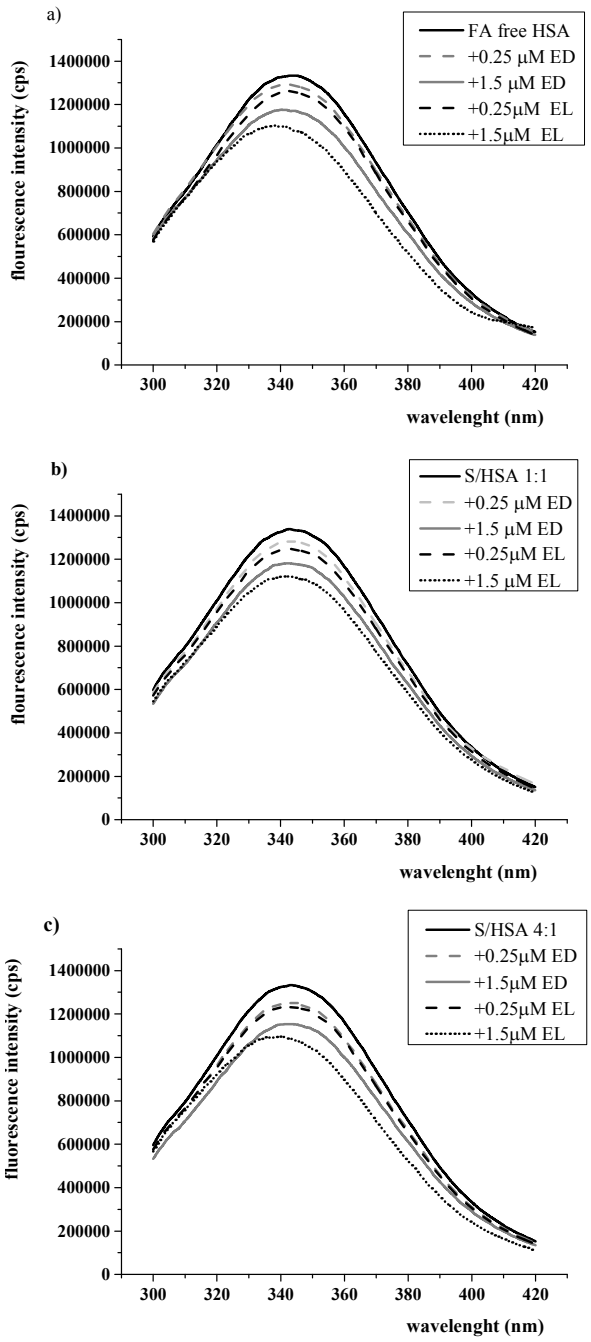

Figure 5. Fluorescence emission spectra of FA-free HSA and S/HSA 1:1 and 1:4 in the presence of different concentrations of EL and ED at 37 °C and pH 7.40. Excitation at 280 nm was used, and emission spectra were recorded in the range from 300 to 420 nm. Concentration of HSA was 0.5 μM, concentrations for each EL and ED were 0.25 μM and 1.5 μM.

After correction of fluorescence intensities of EL and ED at 340 nm for inner filter effect (Materials and Methods, Eq. 1) the Stern-Volmer's constants can be calculated (Materials and Methods, Eq. 2) from the slope of the regression curves $F_o/F-1$ versus [Q] (Figure 6). Obtained Stern-Volmer's plots were linear at all applied conditions, and determined Stern-Volmer's quenching constants (K_{sv}) are shown in Table 2.

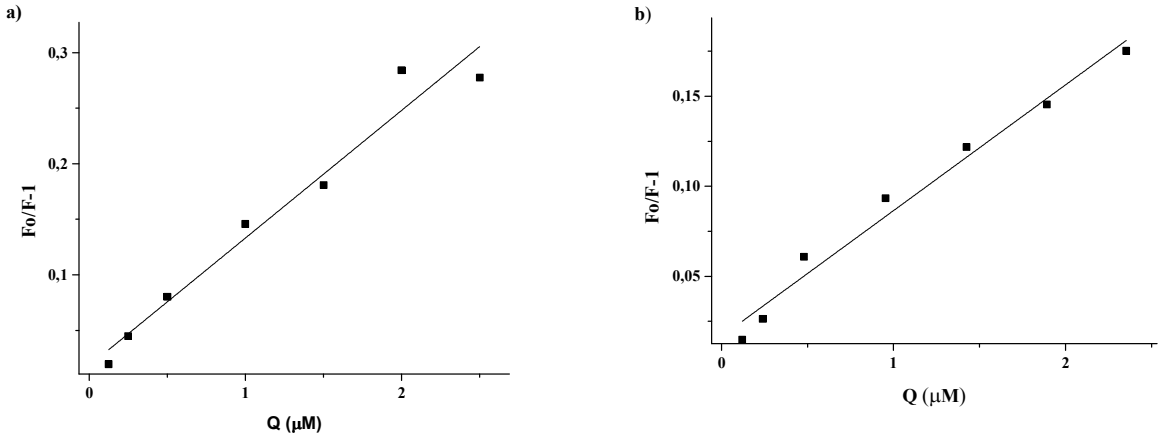

Figure 6. Stern-Volmer's plots: a) EL + FA-free HSA, b) ED + FA-free HSA

Table 2. Stern-Volmer's quenching constants (K_{sv}) and Persons coefficients obtained for titration of 0.5 μM FA-free HSA and S/HSA 1:1 and 4:1 with increasing concentration of quencher (EL and ED) from 0.125 to 2.5 μM (at 37 °C)

Complex	K_{sv} x 10^4 (M^{-1})	r
ENTEROLACTONE		
+ FA-free HSA	11.49	0.985
+ S/HSA 1:1	9.55	0.989
+ S/HSA 4:1	5.87	0.979
ENTERODIOL		
+ FA-free HSA	6.98	0.989
+ S/HSA 1:1	6.95	0.974
+ S/HSA 4:1	7.02	0.984

As average lifetime of biomolecule is 10^{-8} s the quenching rate constants (k_q) are calculated. The obtained k_q values (5.87-11.49 x 10^{12} $M^{-1}s^{-1}$) are two orders of magnitude higher than the limiting diffusion rate constant of the biomolecule (~10^{10} $M^{-1}s^{-1}$) [30]**,** indicating a static type mechanism of fluorophore quenching [38].

3.4. Binding parameters of HSA-enterolignan complexes

Polyphenols are a large and heterogeneous group of phytochemicals present in foods. Lignans are polyphenolic structure phytochemicals with antiestrogenic, antimitotic and anticancer effect [39]. The transport of absorbed phytoestrogens, in particular their binding to specific estrogen carrier proteins (steroid hormone binding protein (SBP) [40] and α-fetoprotein (AFP) [30]) or to nonspecific serum carrier protein, albumin, is important factor determining pharmacodynamics, pharmacokinetics and their biological activities. Phytoestrogens inhibitory effects on the binding of sex hormones to SBP and AFP [40, 30] and unsaturated FA to AFP were reported [30]. Obtained dissociation constants for EL-AFP and ED-AFP complexes were 1.7 x 10^{-5} and 2.2 x 10^{-5} M, respectively [29]. The interactions between flavonoids, phenolic acids, anthocyanins, catechins, resveratol and albumins are reported [18, 41, 42, 43]. In most studies interactions of polyphenols (but no of EL and ED) with HSA were studied using FA-free HSA [42, 43, 44].

The values obtained for binding constants of EL and ED to HSA (Materials and Methods, Eq. 3) were from 1.92 to 11.64 x 10^4 M^{-1} (Table 3), suggesting that the binding between enterolignans and HSA is moderate. These results are also in accordance to the findings that binding constants of polyphenols typically ranged from 10^4 to 10^6 M^{-1} [44]. Soybean isoflavones genistein is naturally occurring estrogen-like molecule with binding constant to HSA of 1.5 x 10^5 M^{-1} [45]. In addition, obtained values show that EL and ED can be stored and transported by HSA as it is the most abundant protein in plasma with concentration of about 0.6 mM versus concentration of specific estrogen carrier protein SBP in nM range [46]. However, EL and ED are mainly presented as glucuronide and sulfate conjugates in body fluids [18]. As the reactions of conjugation with glucuronate and sulfate enable the increase of the solubility and consequently tha elimination of xenobiotics, we considered that EL and ED are in the most extent bound to HSA in unconjugated form. Most studies reported up to now have dealt with native polyphenols, but it was also shown that the polyphenols conjugates (glucuronide and sulfate) circulate bound to albumin [47, 48]. For hydroxycinnamic acid conjugates the affinities to albumin were found to be in the same value of order with aglycons [47]. The changes in the binding activities of quercetin sulfate conjugates depend on the conjugation site [48]. At the same time, recent study demonstrates that β-glucuronidase from human neutrophils is able to deconjugate and thus activate glucuronide conjugates during inflammation (*in vitro*) [49].

Table 3. Binding constants (K_a) and the number of binding sites (n)

Complex	K_a x 10^4 (M^{-1}) 37 °C	n
ENTEROLACTONE		
+ FA-free HSA	11.64	0.896
+ S/HSA 1:1	4.35	0.679
+ S/HSA 4:1	2.43	0.578
ENTERODIOL		
+ FA-free HSA	5.59	0.818
+ S/HSA 1:1	2.50	0.610
+ S/HSA 4:1	1.92	0.507

The highest values of K_a were obtained for FA-free HSA-enterolignans complexes at 37 °C (EL: 11.64 x 10^4 M^{-1} and ED: 5.59 x 10^4 M^{-1}) and the lowest for S/HSA 4:1-enterolignan complexes (EL: 2.43 x 10^4 M^{-1} and ED: 1.92 x 10^4 M^{-1}). Thus, with increased ratio of S/HSA the binding affinity of HSA decreases from 2.7 to 4.8 times for EL and from 2.2 to 2.9 times for ED (Table 3) as well as number of binding sites from 0.896 to 0.578 for EL and from 0.818 to 0.507 for ED.

Simultaneous binding of various ligands to HSA can result in change of their affinity. This can occur when conformational changes of the albumin appear or when ligands occupy the same binding site in serum albumin. Cooperative and competitive interactions between FA and different classes of ligands have been observed in numerous studies [50, 51, 52]. There is no many literature data for influence of FA on polyphenols and phytoestrogens binding to HSA. Panausa et al. [53] found that resveratol binds to HSA and its interaction is modulated by S. The binding constant for resveratol-HSA interaction does not change up to 3-4 molecules of S per HSA molecule, but it markedly decreases at molar ratios S/HSA 5:1 and 6:1. From our results regarding the K_a values for HSA-enterolignans complexes it is evident that S modulates the binding affinity of the HSA to ED and EL, decreasing K_a approximately 2.5 times even in the S/HSA molar ratio 1:1. The increase of S/HSA molar ratio to 4:1 (Table 3) leads to the further significant decrease of binding affinity of the HSA to enterolignans, especially to EL. These findings are very important as 0.1-2 mole of FA is bound to HSA under physiological condition, and the FA/HSA molar ratio can rise above 6:1 in the peripheral vasculature during fasting or extreme exercise [54], or under pathological conditions such as diabetes, liver disease and cardiovascular disease [55].

Modulation of HSA binding affinity to ED and EL could be a consequence of conformation changes of HSA molecules by S, that influence binding of ligands to HSA [50, 51, 52]. On the other hand, ED and EL binding affinity could decrease in the presence of S due to overlapping of two principal drug binding sites with FA binding sites: site I in subdomain IIA overlaps with FA site 7 and drug site II located in subdomain IIIA overlaps with FA sites 3 and 4 [7].

The number of binding site values of enterolignans for FA-free HSA are near one (Table 3). When S/HSA ratio was 1:1 the decreases of values were pronounced. The further decreases caused by FA/HSA ratio 4:1 are much smaller. We found that EL and ED bind to the diazepam binding site (given below). As it was shown by Wong et al. [56] palmitic and oleic acid affect both number of binding sites and binding constants of diazepam to HSA. So, S could have the similar effect on the binding sites number value of EL and ED.

3.5. Site selective binding of enterolactone and enterodiol on HSA

Diazepam has been considered as stereotypical ligand for site II [57]. Therefore, the information about binding of EL and ED to drug site II could be obtained by monitoring of the fluorescence changes of FA-free HSA-EL and FA-free HSA-ED after titration with diazepam. As shown in Figure 7 with addition of diazepam the fluorescence intensity decreases gradually and only FA-free HSA-EL had an obvious blue shift (λ_{max} from 340 to 336 nm) (Figure 7b) indicating that Trp could be placed to more hydrophobic environment after replacement of enterolactone with diazepam.

To facilitate the comparison of the influence of S, EL and ED on the binding of diazepam to HSA the diazepam K_a were calculated using Eq. 3 (Table 4). The results suggested that the binding of S or EL to FA-free HSA led to significant decrease of K_a values for diazepam compared to obtained K_a value for FA-free HSA. In comparison to EL and S, ED showed smaller influence on diazepam K_a. These results suggested that the binding site of EL and ED may be located within site II of HSA. The high affinity binding site for FA is located in subdomain IIIA and FA can influence EL and ED binding as they occupy the same binding place on HSA [7].

Table 4. Binding constants K_a of diazepam and FA-free HSA, FA-free HSA-enterolignans (EL or ED) and S/HSA 1:1 at 25 °C and pH 7.4.

Complex	**K_a x 10^4 M^{-1}**
Diazepam +	
FA-free HSA	18.28
FA-free HSA-EL	2.68
FA-free HSA-ED	12.56
S/HSA 1:1	6.05

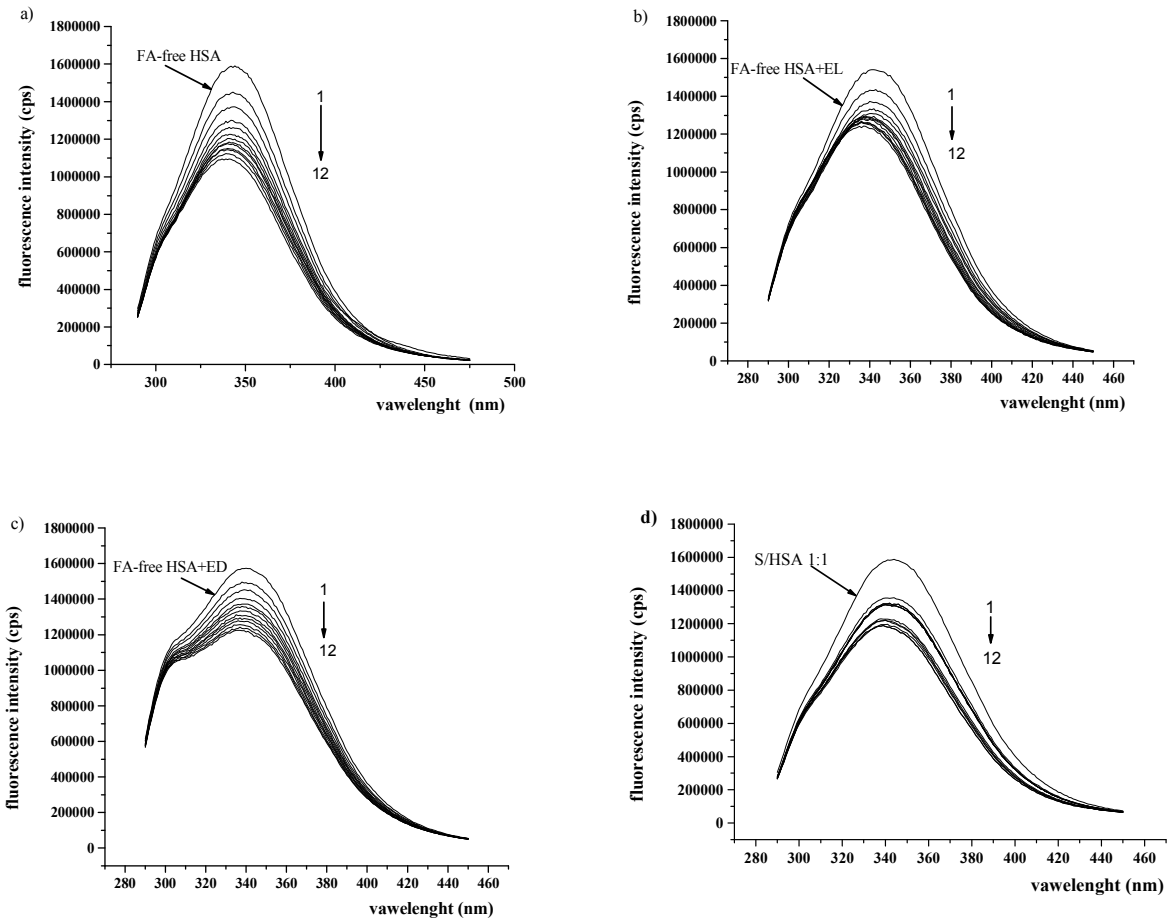

Figure 7. Effect of diazepam to FA-free HSA (a), FA-free HSA-EL (b), FA-free HSA-ED (c) and S/HSA 1:1 (d) at 25 °C and pH 7.4. Excitation wavelength 280 nm, C(HSA) = 0.5 μM, diazepam concentration from 0 to 1.5 μM with an increase of 0.125 μM (curves 1-12).

3.6. Correlation between binding constants and increases of Cys34 reactivity

The highest K_a was obtained for FA-free HSA-EL (Table 3), as well as increase of Cys34 reactivity (Table 1). High correlation (r=0.974) between K_a values and increases of HSA-SH reactivity was found (Figure 8). This result indicated that conformation change in HSA induced upon enterolignans binding is important factor affecting the increase of Cys34 reactivity.

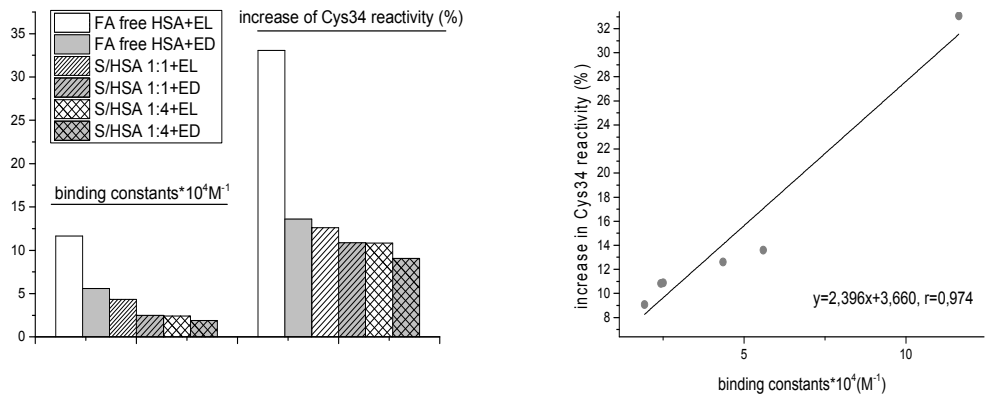

Figure 8. Correlation between binding constants (K_a) and increases of HSA-SH reactivity (%) for FA-free HSA, S/HSA 1:1, 4:1 and enterolignans (EL or ED) complexes.

CONCLUSION

In conclusion, ED and EL increase HSA-SH reactivity, due to HSA conformational changes. They bind moderately to drug site II in subdomain IIIA and K_a values are approximately two times higher for EL compared to ED. Between enterolignans binding constants and changes in the HSA-SH reactivity high correlation was found. This finding could be important for expression of enterolignans antioxidant effect *in vivo* after intake of lignans rich food. Bound S to HSA modulates EL effect on Cys34 reactivity, as well as enterolignans binding to HSA even in the S/HSA molar ratio 1:1. Therefore, in some physiological conditions (intensive exercise, fasting) and some diseases accompanied with increase of non-esterified FA in serum they could significantly modify enterolignans binding to HSA and their effect on HSA-SH reactivity (i.e. antioxidant activity).

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