Binding of quercetin and curcumin to human serum albumin in aqueous dimethyl sulfoxide and in aqueous ethanol

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Abstract
The paper reports the spectrofluorimetric and calorimetric study of binding of two hydrophobic biologically active molecules with antioxidant ability, flavonoids quercetin, and curcumin, to human serum albumin (HSA) in water, aqueous DMSO (0.05 and 0.1 mol. fraction of DMSO), and aqueous ethanol (0.05 mol. fraction of EtOH). Both flavonoids induce the quenching of HSA fluorescence. The stability constants of associates, as well as the changes in enthalpy of the reaction between quercetin and protein, were evaluated. The influence of solvent composition and additions of hydroxypropyl-β-cyclodextrin as a solubilizer of hydrophobic molecules, on the association processes is discussed.

Keywords Albumin · Quercetin · Curcumin · Calorimetry · Binding constant · Enthalpy · Mixed solvents

Introduction
Quercetin (QCT) is a natural flavonol that can be found in plants. It is contained in abundance in apples, honey, raspberries, onions, red grapes, cherries, citrus fruits, and green leafy vegetables [1]. Curcumin (Curc) is a chemical of plant origin (Curcuma longa) as well, used as an herbal supplement, cosmetics ingredient, for food flavoring and coloring. Both compounds are best known for their antioxidant properties. When quercetin reacts with a free radical, it donates a proton and becomes a low-reactive free radical itself stabilized due to the delocalization of electrons by resonance [2]. The antioxidant mechanisms of curcumin include free radicals scavenging as well as modulating the activity of GSH catalase and SOD enzymes active in the neutralization of free radicals; and inhibiting ROS-generating enzymes such as lipoxygenase/cyclooxygenase and xanthine hydrogenase/oxidase [3].

Proposed biological activities and historical use of curcumin-containing plants in the traditional medicine of different cultures [4] have led to numerous attempts to use these two chemicals to treat various diseases. Let us limit to the simultaneous use of quercetin and curcumin for the treatment of cancer [5–7], and some other examples describing the treatment of arthritis [8], obesity [9], and radio-protective prophylaxis [10]. Quercetin, along with its derivatives, has been known to be effective in the treatment of viral diseases, such as SARS-CoV-1 [11], Ebola [12], and even SARS-CoV-2 causing COVID-19 [13]. Quercetin and some other small molecules can bind themselves to the spike protein of the virus, interfering with its ability to infect host cells [11]. Quercetin’s effect on prophylaxis and treatment of COVID-19 is also a subject of the ongoing clinical trial [14], though a small number of participants (50) and neglecting of randomization makes its results questionable.

Both quercetin and curcumin are PAINs (pan-assay interference compounds), i.e., the molecules showing promising but deceptive activities in various biochemical screenings mainly due to unselective interactions with the target [15, 16]. Quercetin and curcumin are also poorly soluble in water [17–20], have low stability (especially curcumin [16, 21, 22]), and bioavailability [16, 20]. One of the ways to enhance the solubility and bioavailability of both compounds and prevent them from decomposing is by encapsulating quercetin or curcumin in the cyclodextrin’s cavity.
[20]. Varying the composition of binary aqueous ethanol or aqueous dimethyl sulfoxide solvent may also benefit the solubility and/or stability. We previously studied the reactions between quercetin, curcumin, and hydroxypropyl-β-cyclodextrin in water–ethanol solvents [23, 24]. Cyclodextrin molecules (CDs) resemble a torus or truncated cone inside it. The inner cavity is hydrophobic while the outer shell is hydrophilic. Due to their structure, CDs are capable of forming inclusion complexes with different hydrophobic organic substrates and improve their solubility. Hydroxypropyl-β-cyclodextrin (HPβCD) and β-cyclodextrin (βCD) are widely used for these purposes. Solvent composition and the host structure should be tuned to guarantee the release of curcumin of quercetin moiety and their binding to the transport protein.

Therefore, the aim of the present paper is to determine the thermodynamic parameters of associates quercetin-human serum albumin (HSA) and curcumin-HSA in aqueous phosphate buffer (pH 7.4), in aqueous dimethyl sulfoxide, and in aqueous ethanol. The formulas of small molecules under study are given in Fig. 1.

**Experimental**

Quercetin (Sigma-Aldrich, China, ≥ 95%), curcumin (Sigma-Aldrich, China, ≥ 65%), dimethyl sulfoxide (EKOS-I, Russia, 99.96%) were used as purchased. Buffer solution with a pH value of 7.4 was prepared using Na2HPO4·12H2O and NaH2PO4·2H2O (Spektr-Khim, Russia). The acidity of the buffer was controlled potentiometrically.

All the solutions for spectral studies were prepared using bidistilled water (κ = 3.6 μS cm⁻¹, pH = 6.6). The ionic strength value I = 0.25 was set due to buffer mixture components. The concentration of HSA (A1653, Sigma-Aldrich) in stock solution stored in the dark place at 4 °C was controlled prior to experiments by UV–Vis spectroscopy (UV-1800, Shimadzu, calibrating plot method) using the known molar extinction coefficient ε280 = 35,700 M⁻¹ cm⁻¹ [25]. Taking into account the poor stability of curcumin [16, 21, 22] and quercetin [26], only solutions prepared directly before experiments were used.

Fluorescence spectra were registered using the CM2203 setup (Solar Ltd., Belarus) at the excitation wavelength λex = 270 nm in the 280–500 nm wavelength range. The excitation and emission slit widths were set at 5 and 2 nm, respectively. The quartz cell with an absorbing layer thickness of 1 cm was used. HSA solution with concentrations of 1.929 × 10⁻⁵ or 1.0756 × 10⁻⁵ mol L⁻¹ and volume of 2.7 mL was placed into the cell and titrated by ten 2 μL injections of dimethyl sulfoxide solution of curcumin (1.976 × 10⁻³ mol L⁻¹) or ethanol solution of quercetin (2.197 × 10⁻³ mol L⁻¹).

Following the recommendations [27–29], all the registered emission spectra of HSA “guest molecule” mixtures (Fig. 2a, c) were corrected for the inner filter effect using the equation:

\[
F_{\text{cor}} = F_{\text{obs}} \times 10^{0.5(A_{\text{ex}}+A_{\text{em}})}
\]

where \(F_{\text{obs}}\) and \(F_{\text{cor}}\) are the observed and corrected relative intensities of emission, \(A_{\text{ex}}\) and \(A_{\text{em}}\) are the differences in the absorbance values of the sample upon the addition of the ligand at the excitation (270 nm) and emission (280–500 nm) wavelengths, respectively. The absorbance values for quercetin and curcumin solutions were determined using calibrating plot method in the blank experiment. The UV–Vis data are in good agreement with the literature values [30].

Since the solubility of quercetin in water is low, and curcumin solubility is extremely poor, the dimethyl sulfoxide or ethanol solutions of compounds were used. Therefore, we...
also checked if the observed changes in the luminescence intensity are caused by DMSO or ethanol additions toward protein. The experiments included titration of 2.7 mL of 1.929·10⁻⁵ or 1.0756·10⁻⁵ mol L⁻¹ HSA in phosphate buffer (pH 7.4) by pure non-aqueous solvent. The observed changes in the emission spectrum of albumin were within the error limits of the spectrofluorimeter; therefore, curcumin or quercetin presence was responsible for the alteration of the fluorescence spectra. The maximum mole fraction of the non-aqueous solvent at the end of titration was ca. 0.002.

Similarly, the influence of cyclodextrins on the fluorescence spectra of HSA was checked. The aliquot of 2.7 mL of 1.929·10⁻⁵ mol L⁻¹ HSA in phosphate buffer (pH 7.4) was titrated against the buffer solution of β-cyclodextrin (βCD, 1.910·10⁻⁵ mol L⁻¹). There were ten injections of 10 µL each. No changes in the emission spectrum of protein were detected within the error limits of determination. Therefore, no interaction between HSA and βCD occurs under the studied experimental conditions. Our results are in good correspondence with the reported data on cyclodextrins (CD)-bovine serum albumin (BSA) and CD-HSA interactions [31–33]. Authors [31–33] specify that the constants of CD-protein association are not to exceed 10³, which makes the yield of complexes negligible under our experimental conditions.

To calculate the stability constants of quercetin-HSA, curcumin-HSA associates, the spectral data were processed using KEV software [34].

The optimization procedure is based on varying the binding constant until the minimum value of:

$$M = \sum_{i=1}^{K} (F_{\lambda_{\text{max}}}^{\exp} - F_{\lambda_{\text{max}}}^{\text{calcd}})^2$$

(2)

is reached, where $M$ is a cost function, $K$ is a number of experimental points $F_{\lambda_{\text{max}}}^{\exp}$ and $F_{\lambda_{\text{max}}}^{\text{calcd}}$ are the experimental and calculated fluorescence intensities at the wavelength corresponding to the maximum fluorescence. $F_{\lambda_{\text{max}}}^{\text{calcd}}$ is calculated as follows:

$$F = \sum_{j=1}^{N} F_j X_j$$

(3)

where $F_j$ is a partial fluorescence of the $j$th component, $X_j$ is a mole fraction of the $j$th component, $N$ is a number of components (reagents and products). Partial molar properties are found using the linear regression equation.

ITC experiments have been provided by the use of PEAQ ITC (Malvern Pananalytical) calorimetric equipment. HSA solutions for ITC experiments were prepared from the stock protein solution in buffer and used 1 h after dilution in the buffer-DMSO mixture (5–8 vol.% of DMSO). DMSO contents did not exceed 8% to prevent HSA conformational rearrangements monitored by changes in UV–Vis light absorption spectra. Quercetin solutions for ITC experiments were prepared from the stock quercetin solution in DMSO. After preparation of quercetin solution in buffer-DMSO mixture (5–8 vol.% of DMSO), the solution was centrifuged at 10,000 g to delete possible aggregates. The supernatants were taken for ITC. HSA and quercetin solutions at a concentration of 5–8% of DMSO were defined by UV–Vis spectroscopy previously to be used in the ITC experiments. It was checked that the stability of HSA and quercetin solutions was maintained for hours at a concentration of 5–8% of DMSO after these preparation steps.

PEAQ ITC experiments have been implemented under the following conditions: (1) 0.005–0.015 mM of HSA was in the cell, and 0.100–0.330 mM quercetin was in a syringe; (2) 0.020 mM quercetin was in the cell, and 0.137–0.270 mM of HSA was in a syringe. Similar concentration conditions were followed for blank experiments. The equivalent concentration of 5–8% of DMSO was taken for solutions prepared for syringe and cell. In the literature for obtaining ITC data, both experimental procedures are used: quercetin titrating to serum albumin [35–37] and serum albumin titrating to quercetin [37]. Therefore, ITC experiments and further calculations on HSA-quercetin binding have been implemented under the following conditions: 0.015 mM of HSA was in the cell, and quercetin (0.330 mM) was in a syringe at 8 vol.% DMSO. The blank titrations have been carried out for all the cases.

The dependences of enthalpic effects of subsequent injections of titrant into the cell are presented in Fig. 2.

Thermodynamical binding parameters have been estimated with the use of an Independent binding model. The calculations were realized in the frame of Launch Nanoanalyse software included to the Nano ITC 2G calorimeter by TA Instruments Co. in the SPbSU Science Park, Center for Thermogravimetric and Calorimetric Research. Enthalpy of interaction was determined directly from the calorimetric experiment.

**Results and discussion**

The examples of spectrofluorimetric titrations are given in Fig. 3.

The calculations of stability constants from spectrofluorimetric data using KEV are advantageous (comparing to the simple double reciprocal plotting) since they allow checking the different stoichiometry of the products and taking into account the intrinsic fluorescence of associates. The stoichiometric model suggesting a 1:1 reaction between small molecule and protein gives the best fit of the experimental results.
The calculated stability constants are given in Table 1, along with the literature values.

The errors of experimentally determined constant values in Table 1 are the half-width of the confidence intervals at the confidence level of 0.95 and 3–5 parallel experiments.

It is worth noting that we also performed a UV–Vis titration study of the HSA-curcumin interaction in the water. However, the spectra resulting from the graduate adding of curcumin to the protein solution were extremely close to the UV–Vis spectra of free curcumin with the corresponding concentration. The same problem was noticed in the paper [27]. It made the calculations unstable and low-reliable, though the stability constant value of \( \log \beta = 4.63 \pm 0.12 \) (five replications) at pH 7.5 (3.90 ± 0.27 at three replication and pH 6.0) are in somewhat agreement with more reliable spectrofluorimetric data (Table 1).

Moreover, the calorimetric titration of quercetin against HSA also returned the value of \( \log K_b = 5.75 \) confirming the strong affinity of the small molecule to the protein. However, the calorimetric measurements revealed the stoichiometry of the reaction to be 0.2 mol of quercetin per 1 mol of protein. The probable reasons are (1) the steric unavailability of the most of potential binding sites caused by the complex spatial structure of protein and by the undetected HSA aggregation in buffer-DMSO solutions, and (2) some aggregation of quercetin due to its limited solubility in buffer-DMSO solutions [48, 49]. The experimental difficulties related to the measurements of heats of quercetin binding to HSA allow only the estimation of the change in enthalpy of the reaction between quercetin and protein. The value determined in the present paper is \( \Delta H_t = -113 \pm 20 \text{ kJ mol}^{-1} \).

The values of binding constants of quercetin to serum albumins are close to each other, except for the results of the paper [40], which is clearly an overestimation. The analysis of the existing literature showed a significant difference in the reported stability constants of albumins-curcumin associates. The only thing most of the authors agree on is the stoichiometry being 1:1 in the case of serum.
We believe that the most significant reason for the observed discrepancies in the literature is the low stability of curcumin, which forces quick experimental performance to avoid solvolysis [16]. Another way of curcumin degradation is the photolysis; however, since the maxima of excitation and emission do not coincide with the absorbance maximum of curcumin ($\lambda_{\text{max}} = 430$ nm), bleaching is likely avoided in the spectrofluorimetric measurements.

We suppose our stability constants values for the albumin-curcumin complex to be reliable since all the necessary precautions while working with curcumin were taken.

The addition of a small amount of either DMSO or ethyl alcohol does not impact the binding constant of quercetin to HSA (Table 1). Further increasing of the DMSO content leads to protein denaturation [49]. We also observed significant HSA denaturation in aqueous ethanol even at low concentrations of alcohol, which is in agreement with literature data [50]. However, small DMSO addition increases the binding constant of curcumin to HSA (Table 1), which is probably related to the better solvation of curcumin and, especially, their associate. Further increasing of DMSO content leads to a decrease of the association constant, which may reflect the stronger competing for curcumin between protein and a non-aqueous solvent.

In addition to the experiment described above, the titration of HSA solution (2.6 mL, $9.8 \times 10^{-6}$ mol L$^{-1}$) against the mixture of quercetin ($1.791 \times 10^{-3}$ mol L$^{-1}$) and hydroxypropyl-\(\beta\)-cyclodextrin ($9.217 \times 10^{-3}$ mol L$^{-1}$) was performed (ten injections, 2 $\mu$L each). Cyclodextrin does not interact with HSA as was found in the separate preliminary experiment; however, it binds quercetin with $\lg \beta = 3.46$ as was found spectrophotometrically before. The processing of the results of mixture titration, taking into account the host–guest association between cyclodextrin and quercetin, has returned the same value of $\lg \beta_{\text{quercetin-HSA}} = 5.74 \pm 0.09$ (3 parallel experiments).

### Conclusions

The quenching of human serum albumin fluorescence in the presence of increasing amounts of quercetin and curcumin was studied. The binding constants of both compounds to HSA were calculated from the spectrofluorimetric and calorimetric titration data. Quercetin binds strongly to protein with $\lg K_b = 5.7–5.8$ units, while curcumin forms much less stable associates with $\lg K_b = 4.3–4.7$ units. The addition of either 5 or 10 mol % of DMSO or 5 mol % of ethanol toward an aqueous solution of quercetin and HSA does not lead to a significant change in the binding constant of quercetin to HSA.
affect the value of the association constant. However, small DMSO addition increases the binding constant of curcumin to HSA, which is probably related to the better solvation of curcumin and, especially, its associates. Further increasing of DMSO content leads to a decrease of the association constant, which may reflect the stronger competition for curcumin between protein and a non-aqueous solvent. The change in enthalpy of quercetin binding to HSA was estimated to be $\Delta H^o = -113 \pm 20 \text{ kJ mol}^{-1}$.

It was confirmed that hydroxypropyl-$\beta$-cyclodextrin does not interact with HSA. The system containing protein, quercetin, and hydroxypropyl-$\beta$-cyclodextrin is best described with two chemical processes of HSA + quercetin and hydroxypropyl-$\beta$-cyclodextrin + quercetin. Thus, protein and macrocycle only compete for quercetin; the inclusion complex is unable to bind to HSA.

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Authors contributions Conceptualization contributed TU, GG, and VS; Methodology contributed TU, GG, AB, and YA; Formal analysis and investigation contributed GG; AB; YA; AS; DA; NK; Writing—original draft preparation contributed TU, GG, and AB; Writing—review and editing contributed TU and GG; Funding acquisition contributed TU and GG; Resources contributed TU, AB, YA, and AS; Supervision contributed TU, GG, and VS.

Declarations

Conflict of interest The author’s declared that they have no conflict of interest.

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