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Original scientific paper

3-Hydroxyflavone–bovine serum albumin interaction in dextran medium

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Abstract: A bioactive flavonol, 3-hydroxyflavone (3-HF), in systems based on dextran 70 (Dx70, an important bio-relevant polysaccharide) and bovine serum albumin (BSA, a carrier protein) was studied by fluorescence and circular dichroism. Changes produced by different concentrations of Dx70 on the fluorescent characteristics of 3-HF and on the excited-state intramolecular proton transfer (ESIPT) process were studied. The influences of 3-HF binding and of Dx70 on the secondary structure of BSA were investigated by circular dichroism spectroscopy. The influence of temperature (30–80 °C range) on the intrinsic tryptophan fluorescence in 3-HF/BSA/Dx70 systems was investigated. The results are discussed with relevance to 3-HF as a sensitive fluorescence probe for exploring flavone–protein interactions in plasma expander media and for its biological evaluation.

Keywords: flavones; proteins; dextran 70; fluorescence spectroscopy; ESIPT.

INTRODUCTION

Flavones and related compounds of the flavonoid group in plant polyphenolic compounds have various therapeutic properties, such as: antioxidant, antiradical, angioprotective, making them effective agents against cancers, tumors, cardiac problems, inflammations, allergies and acquired immune deficiency syndrome.[1–6] Other remarkable types of activity of flavones are based on their dual fluorescence behavior, known as systems exhibiting excited state intramolecular proton transfer, ESIPT, which is useful for exploring the structure, function, dynamics, interactions and microenvironment in biological systems, *e.g.*, proteins.[7–12]

The interaction between flavonoid compounds and serum albumins were reported.[9,13–22] Bovine serum albumin (BSA) represents the major globular protein of bovine blood plasma. BSA is structurally a polypeptide chain containing

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582 amino acids residues, which, due to its relatively high aqueous solubility, binds to several types of biological molecules and thus determines physiological function.[23,24] The two tryptophan (Trp) residues in the BSA structure are responsible for its intrinsic fluorescence: Trp212 that belongs to subdomain IIA within a hydrophobic binding pocket and Trp134 that belongs to the first subdomain IB, located on the surface of the albumin molecule. Based on this, fluorescence spectroscopy is a suitable tool to monitor changes in the intrinsic fluorescence of BSA on binding of various ligands.[25]

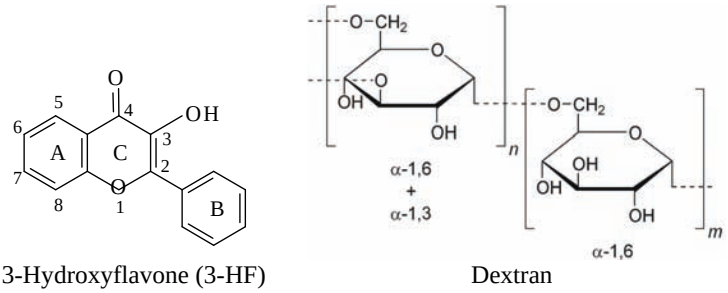
Dextran is a family of microbial 1→6–α–D–glucans derived from *Leuconostoc mesenteroides* with varying proportions of other types of linkages, (1→2)-α-, (1→3)-α- and (1→4)-α-branch linkages, which is used as plasma expander.[26] Due to its solubility in water and organic solvents, dextran is a versatile biomacromolecule for preparing nanofibrous electro-spun membranes for biomedical applications.[27] In line with this, it was found that BSA and lysozyme were directly incorporated into an electro-spun dextran membrane without compromising its morphology.[27] According to Jung *et al.*, depending on the ratio of dextran to BSA used, about 0.5–1 mol of dextran could be bound to 1 mol of native BSA, indicating that the BSA surface in the conjugates was covered with dextran without disruption of its native conformation.[28]

This study deals with an analysis of the bioactive flavonol, 3-hydroxyflavone (3-HF) in systems based on dextran 70 (Dx70) and bovine serum albumin (BSA) (a carrier protein), by fluorescence and circular dichroism spectroscopy.

EXPERIMENTAL

Materials

A stock solution of 3-hydroxyflavone (3-HF, Sigma) was prepared in methanol (spectrophotometric grade, Sigma). Aliquots from stock solution were used to prepare the working solutions of concentration 6×10^{-5} M. A stock solution of dextran 70 (Dx70) was prepared in distilled water, with working concentrations in the range 0.33–1.66 μM. The molecular structure of 3-HF and Dx70 are shown in Scheme 1. Bovine serum albumin (BSA) was purchased from Merck, Darmstadt. Phosphate buffer used was 0.1 M, pH 7.4. A phosphate buffer was purchased from Sigma–Aldrich, Steinheim, Germany.



3-Hydroxyflavone (3-HF) Dextran

Scheme 1. Molecular structure of 3-hydroxyflavone (3-HF) and dextran.

Methods and apparatus

The fluorescence emission and excitation spectra were recorded with a Jasco FP-6500 spectrofluorometer, using 3 nm bandpasses for the excitation and the emission monochromators, a detector response of 1 sec, data pitch of 1 nm, a scanning speed of 100 nm min^{-1}. The excitation wavelengths were 365 nm (for hydroxyflavone emission) and 280 nm (for Trp emission of the BSA protein).

The fluorescence lifetime decays were recorded in a time-correlated, single photon counting FLS920 system from Edinburgh Instruments, with laser excitation at 375 nm, a lifetime scale of 100 ns and 2048 channels. The laser had a width of 1.5±0.5 nm, and a pulse duration of 50 ps. The data were fitted with a multi-exponential decay (reconvolution) and the accuracy of the fit was checked on grounds of χ^2, which was less than 1.2. Intensity-averaged lifetimes were calculated according to the equation:[29]

$$\langle\tau\rangle = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i}; \quad f_i = \frac{\alpha_i \tau_i}{\sum_i \alpha_i \tau_i} \quad ; \quad a_i = \frac{\alpha_i}{\sum_i \alpha_i}$$

The circular dichroism spectra were measured on a Jasco J-815 spectropolarimeter in the wavelength range of 200–300 nm. The experimental data was fitted with DichroWeb[30] in order to obtain the secondary structure with the K2D neural network algorithm.[31]

RESULTS AND DISCUSSION

Steady-state fluorescence analysis

The fluorescence emission spectra of 6×10^{-5} M, 3-HF in the presence of Dx70, for an excitation wavelength, λ_{ex} = 365 nm, is shown in Fig. 1A. Two fluorescence emission bands were observed: a broad band at 470 nm, which corresponds to the normal (N*) form (S_1 (π–π^*) $\rightarrow$ S_0, where no proton transfer process occurs) and at 528 nm, attributed to the tautomer (T*) form, which is generated from an excited-state intramolecular proton transfer (ESIPT) process of the internal H-bond, C(4)=O···HO–C(3).[7] No significant changes in emission wavelengths for the N* and T* forms were observed with increasing concentration of DX70, but the fluorescence intensity of the bands increased slightly. In the fluorescence excitation spectra of 3-HF, monitored at 530 nm, of 3-HF in the presence of Dx70 and in direct comparison with 3-HF at pH 7.4 (Fig. 5B), two bands at λ_{ex} around 304 and 350 nm were observed, which are attributed to the absorption of 3-HF. According to Schipfer *et al*., the UV-absorption spectra of flavones are characterized by two absorption bands (π–π^*), Band I (300–380 nm) attributed to the absorption by the cinnamoylic portion (B+C rings) and band II (240–285 nm), by the benzoyl portion (A+C rings) of flavornoid molecules.[32]

Addition of BSA (Fig. 1C) leads to a 5-nm red-shifted fluorescence emission of the N* form, λ_{em} = 475 nm, and a 4-nm red-shifted fluorescence emission of the T* form, λ_{em} = 532 nm. As could be observed, the fluorescence intensity of both forms increased with increasing concentration of BSA. It could be noticed that the T* emission was higher than the N* emission, indicating an efficient

excited-state proton transfer process. In addition, with increasing BSA concentration, the intensity ratio of N*:T* fluorescence (I_{N*}/I_{T*}) decreased from I_{N*}/I_{T*} = 0.625 for a concentration of 0.33 μM BSA to I_{N*}/I_{T*} = 0.406 for 1.66 μM BSA. This feature was attributed to the more hydrophobic environment of 3-HF in the presence of BSA. Moreover, a high affinity binding of 3-HF on BSA in the presence of Dx70 was considered. The fluorescence excitation spectra of 3-HF, monitored at 530 nm, in the presence of Dx70 with varying BSA concentrations is presented in Fig. 1D. Comparing the spectra in Fig. 1D with those in Fig. 1B, the bands with λ_{ex} in the region 285–293 nm are attributed to π–π* transitions of the aromatic amino acid residues, Tyr and Trp.[33,34] The broad band at $\lambda_{ex} \approx 312$ nm may be due to 3-HF binding to the BSA structure, while the band in the region 353–372 nm corresponds to the absorption of 3-HF. The band with $\lambda_{ex} \approx 419$ nm, attributed to the anion form 3-HF,[12] was 3 nm red-shifted ($\lambda_{ex} \approx 422$ nm) with increasing BSA concentration. These features are due to specific interactions of 3-HF anion form with the environment of amino acid residues in the BSA structure.

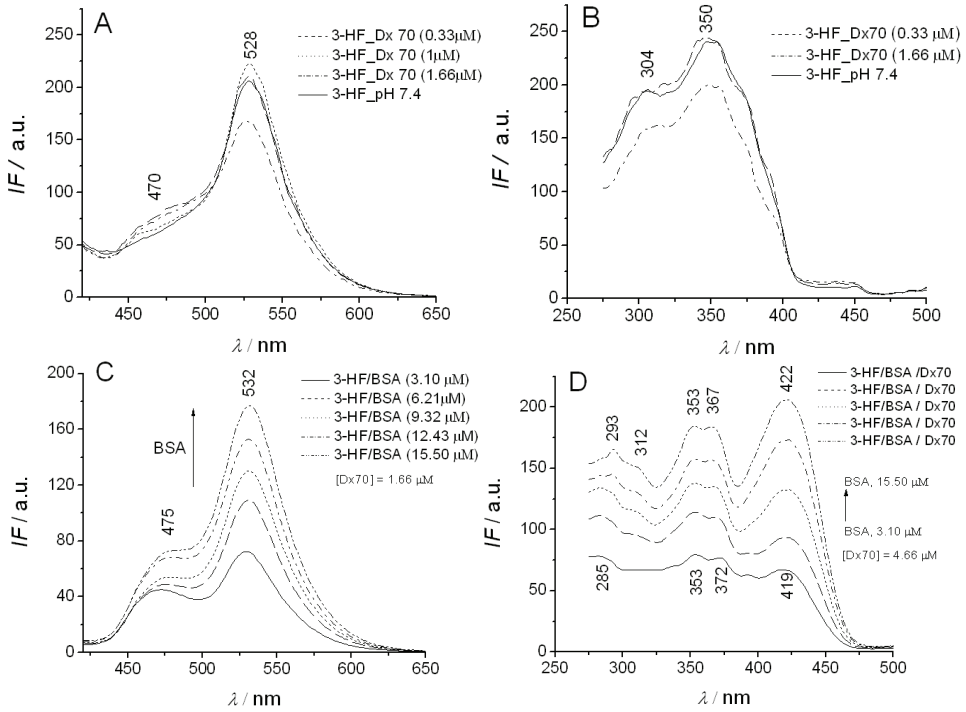


Fig. 1. Fluorescence emission spectra of 6×10^{-5} M 3-HF in Dx70 medium without (A) and with BSA(C), λ_{ex} = 365 nm, and their corresponding excitation spectra (B and D); λ_{em} = 530 nm.

The influence of temperature on intramolecular ESPT

The influence of temperature in the range of 30–80 °C on the excited-state intramolecular proton transfer (ESIPT) tautomer emission of 3-HF in the presence of BSA and Dx70 at pH 7.4 for an excitation wavelength of 365 nm, is shown in Fig. 2A. No changes in the fluorescence emission wavelengths of the N* (λ_{em} = 475 nm) and T* (λ_{em} = 532nm) forms were observed but their intensities decreased with increasing temperature. With gradual increase in temperature, the intensity ratio I_{N*}/I_{T*} decreased, Table I, providing a less hydrophobic environment of 3-HF, in that the internal H-bonds at the binding sites in BSA are weak. Without Dx70, it was found that the intensity ratio I_{N*}/I_{T*} of 3-HF increased, a fact corresponding to the more hydrophobic environment of 3-HF, Table I. The intramolecular ESPT tautomer emission of 3-HF in the presence of BSA and Dx70 was notably even when the temperature increased. From the excitation spectra in Fig. 2B, it could be seen that with increasing temperature, the bands at $\lambda_{ex} \approx 293$ nm, characteristic for Tyr and Trp absorption, strongly decrease at 80 °C and a broad absorption band appeared around 306 nm. The broad band around 357 nm corresponds to 3-HF absorption. This is indi-

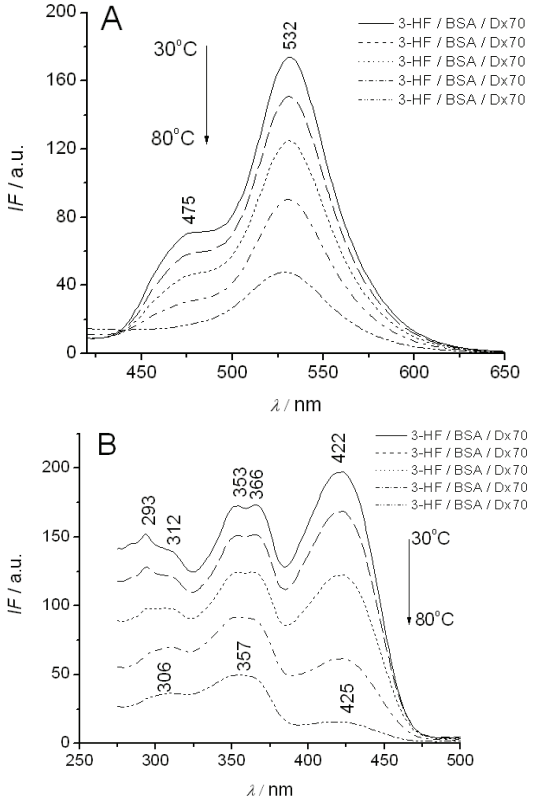


Fig. 2. A) Fluorescence emission and B) excitation spectra of 3-HF/ /BSA interaction in the presence of Dx70 at pH 7.4 *vs.* temperature: Dx70:BSA = 1:1 (*V*/*V*); [3-HF] = 6×10^{-5} M; λ_{ex} = 365 nm, λ_{em} = 530 nm.

cative of 3-HF binding to BSA, especially at the site in the proximity of the Trp 212 residue. The band at λ_{ex} = 422 nm was attributed to the absorption of the anion form of 3-HF[12] and as the temperature increased, a slight bathochromic shift to 425 nm was registered at 80 °C. A slight denaturation of BSA at this temperature should also be taken into consideration.

TABLE I. Fluorescence characteristics of the 3-HF/BSA interaction with and without Dx70, in phosphate buffer pH 7.4 (PBS). λ_{N*} and λ_{T*}, position of the fluorescence emission maxima of the normal (N*) and tautomer (T*) forms; λ_{ex} = 365 nm

System	t / °C	λ_{N*} / nm	λ_{T*} /nm	I_{N*} / I_{T*}
3-HF / BSA / PBS	25	471	530	0.460
	30	475	530	0.469
	40	475	530	0.477
	50	475	530	0.495
	60	475	529	0.525
	80	475	528	0.545
3-HF / BSA / Dx70 / PBS	25	476	532	0.406
	30	476	532	0.402
	40	476	531	0.381
	50	476	531	0.368
	60	474	531	0.333
	80	–	528	0

The influence of temperature on the intrinsic fluorescence emission of BSA

It is known that BSA has two Trp residues involved in its intrinsic fluorescence emission: Trp212, in a hydrophobic binding pocket of the subdomain IIA, and Trp134, belonging to the first subdomain IB, on the surface, more exposed to hydrophilic environment.[35] The fluorescence emission of the native BSA at an excitation wavelength of 279 nm, is 340 nm,[28] and changes in the intrinsic fluorescence of BSA may occur in dependence on the environment of the Trp residues.

Intrinsic tryptophan fluorescence emission spectra of 3-HF binding and Dx70 on the BSA structure at pH 7.4 for an excitation wavelength λ_{ex} = 280 nm are shown in Fig. 3A. It was registered that with increasing BSA concentration, the fluorescence intensity of Trp gradually increased, but with no significant emission shifts λ_{em} to 334–336 nm. Thus, 3-HF in the presence of Dx70 does not cause conformational changes in the secondary structure of BSA.

The secondary structure of the BSA undergoes conformational changes under thermal treatment. The helicity of the protein (66 %) decreased with rising temperature; half of the original helicity was lost at 80 °C, while a helicity of 16 % was still maintained even at 130 °C.[36] As the temperature increases, Fig. 3B, an 8-nm blue-shift in the fluorescence emission was observed, λ_{em} = 327 nm,

and the feature corresponds to a more hydrophobic environment of the Trp 212 residue when 3-HF binds to BSA.

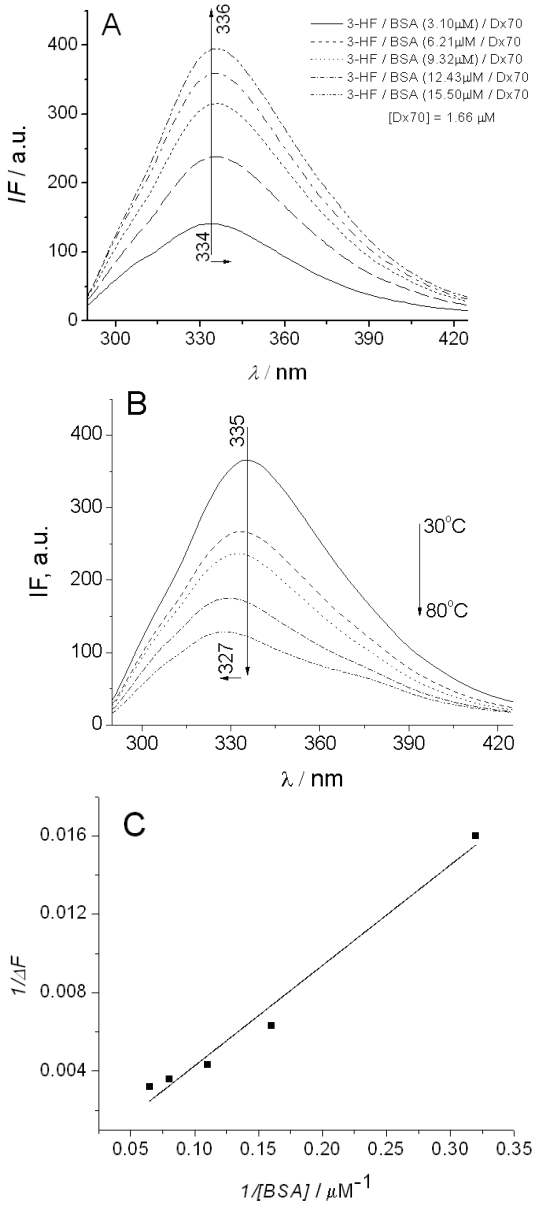


Fig. 3. Tryptophan intrinsic fluorescence emission spectra of 3-HF binding and Dx70 on the BSA structure, A) at different BSA concentrations and B) at different temperatures at Dx70: BSA = 1:1 (*V*/*V*), pH 7.4 and λ_{ex} = 280 nm. C) The variation $1/\Delta F$ of Trp fluorescence emission as a function of the molar concentration of BSA.

The binding constant between 3-HF and BSA in Dx70 medium could be estimated from the fluorescence emission data using a modified Benesi–Hildebrand equation, as follows:[37]

$$\frac{1}{\Delta F} = \frac{1}{\Delta F_{max} K [\mathrm{BSA}]} + \frac{1}{\Delta F_{max}}$$

where $\Delta F = F_x - F_0$, whereby F_x and F_0 represent the Trp fluorescence intensities in the presence of Dx70 at pH 7.4 and in the absence of Dx70, respectively. ΔF_{max} is the maximum change in the Trp fluorescence intensity and K is the binding constant. The variation $1/\Delta F$ for the intrinsic Trp fluorescence emission intensity for various molar concentrations of BSA is presented in Fig. 3C, from which the K value was estimated to be 5.13×10^4 M^{-1} (SE = 7.17×10^{-4}, r^2 = 0.975). Thus, a high binding affinity of 3-HF to BSA in the presence of Dx70 was registered. For human serum albumin (HSA), Sytnik and Litvinyuk[12] reported that the higher affinity site of HSA has an association constant of $K = 7.2\times10^5$ M^{-1} and predominantly hosts the 3-HF anion, while the normal tautomer has a lower affinity, $K = 2.5\times10^5$ M^{-1}.

Time-resolved fluorescence analysis

Lifetime measurements on the 3-HF band were performed in order to see the effect that the presence of Dx70 has on the species present in solution. The results are presented in Table II and the decay profiles in Fig. 4. When bound with BSA, 3-HF had three lifetime components, as was found in pH 7.4 phosphate buffer solution (PBS) as well. In order of increasing lifetime, the three components were attributed to the tautomer, T* (0.18 ns), anion, A* (1.46 ns) and normal, N* (5.39 ns), species of 3-HF.[38] Lifetime measurements can also yield information on the relative population of these species and the changes that occur upon interaction with BSA.

TABLE II. Lifetimes (τ / ns), fractional intensities (f / %) and relative populations (a / %) of 3-HF excited species under different experimental conditions

System	τ_1	τ_2	τ_3	f_1	f_2	f_3	a_1	a_2	a_3	$\langle\tau\rangle$
PBS	0.18	1.46	5.39	44.39	31.60	24.02	90.20	8.16	1.63	1.82
BSA	1.03	4.11	8.08	13.92	47.74	38.34	45.13	38.94	15.93	5.21
BSA/Dx70	1.07	3.75	7.94	20.34	47.55	32.11	53.08	35.38	11.54	4.58

In the presence of BSA, all the lifetimes increased to 1.03, 4.11, 8.08 ns, respectively, and the average lifetime increased as well from 1.82 to 5.21 ns. The relative population of the T* form decreased two-fold, from 90.20 to 45.13, while both the anion and the normal species increased, which determined a similar trend for the fractional intensities. As both T* and N* are tautomers of the neutral molecule, the anion increased from 8.16 to 38.94 % in BSA, at the expense of neutral 3-HF, which decreased from 91.84 to 61.96 %. This means that deprotonated 3-HF is favored as the binding species. A third effect of binding to BSA was perturbation of the excited state processes, as the T*/N* popul-

ation ratio dramatically decreased from 55.34 to 2.83 and the intensity ratio from 1.85 to 0.36.

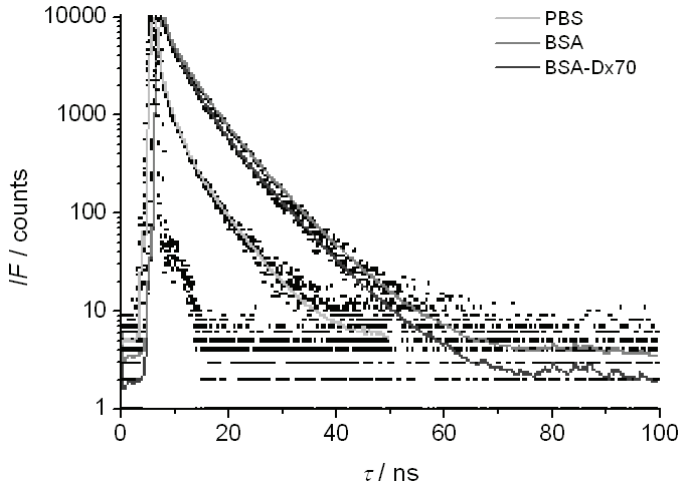


Fig. 4. Decay profiles for 3-HF in PBS, BSA and BSA in the presence of Dx70: (○) – instrument response; (●) – experimental data; lines – fitted data.

The addition of Dx70 had a slight tendency of reverting the effect that BSA had on 3-HF, *i.e.*, a slight decrease in the lifetimes to 1.07, 3.75 and 7.94 ns, respectively, and of the average lifetime to 4.58 ns, on one hand, and on the other a slight decrease of the deprotonated 3-HF to 35.38 %, while the neutral molecule percentage rises to 64.62 %. The reverting tendency is valid also for the excited state processes, as the population ratio becomes 4.60 and the intensity ratio 0.63.

Circular dichroism analysis

A circular dichroism spectrum of a protein can be considered the sum of the characteristic individual spectra of each type of secondary structure present in the protein. Thus, by deconvolution, the contents of each of the secondary structures of proteins can be determined. The data in Table III show that binding of 3HF decreased the α-helix percentage by 6 %, from 59 to 53 %, increasing thereby the content of the random coil structure the most, as is the case with most ligands. On the other hand, Dx70 increased the α-helix percentage by 3–5 % at the expense of both the β-sheet and random coil structures, both in the absence and in the presence of the ligand. It can be seen that bound BSA in the presence of Dx70 reverts to a structure very similar to the native one, 58, 8 and 34 % for the content α-helix, β-sheet and random coil, respectively, compared to 59, 8 and 33 %, respectively, for the native protein. Thus, Dx70 had the effect of renaturation on the serum albumin.

TABLE III. The effect of 3HF binding and Dx70 on the secondary structure of BSA; *NRSMD*: normalized root mean square deviation

System	α-helix, %	β-sheet, %	Random coil, %	*NRMSD**
BSA	59	8	33	0.109
BSA /Dx70	62	6	31	0.185
3-HF /BSA	53	10	37	0.187
3-HF /BSA /Dx70	58	8	34	0.202

The reverting effect of Dx70 on both BSA in the presence of the ligand and on 3-HF bound to BSA may be due to both a competitive interaction of the 3-HF to BSA and Dx70 and to slight structural modifications that the presence of Dx70 induced on the protein that increased the percentage of the α-helix structure.

CONCLUSIONS

In Dx70 medium and with gradual increase in the temperature, the intensity ratio of N* and T* fluorescence (I_{N*}/I_{T*}) in the 3-HF / BSA systems decreased, leading to a less hydrophobic environment of 3-HF, because the internal H-bonds at the binding sites in BSA are weak.

In Dx70 medium, 3-HF binds to BSA especially at the site in the proximity of Trp 212 residue. The value of binding constant, 3-HF to BSA in the presence of Dx70 was $K = 5.13\times10^4$ M^{-1}.

Dx70 increased the α-helix percentage by 3–5 % at the expense of both the β-sheet and random coil structures, both in the absence and in the presence of 3-HF. It was observed that the bound BSA in the presence of Dx70 reverted to a structure very similar to the native one, 58, 8 and 34 % for α-helix, β-sheet and random coil, respectively, compared to 59, 8 and 33 %, for the native protein. Thus, Dx70 had an effect of renaturation on the serum albumin.

The data have relevance in future studies concerning flavone–protein interaction with 3-HF as a sensitive fluorescence probe in plasma expanders media, as well as in the future studies concerning its biological evaluation.

ABBREVIATION LIST

3-HF – 3-Hydroxyflavone
Dx70 – Dextran 70
BSA – Bovine serum albumin
Trp – Tryptophan
ESIPT – Excited-state Intramolecular Proton Transfer
N* and T* – Normal and tautomer forms
I_{N*}/I_{T*} – The intensity ratio of N*:T* fluorescence

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ИЗВОД

ИНТЕРАКЦИЈА 3-ХИДРОКСИФЛАВОН–ГОВЕЂИ СЕРУМСКИ АЛБУМИН У МЕДИЈУМУ ДЕКСТРАНА

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Спектроскопска анализа биоактивног флавонола, 3-хидроксифлавона (3-HF), у систему базираном на декстрану 70 (Dx70, важан био-релевантан полисахарид) и говеђег серумског албумина (BSA, протеин носач), изведена је применом флуоресценције и циркуларног дихроизма. Студиране су промене настале применом различитих концентрација Dx70 на флуоресцентне карактеристике 3-HF, као и побуђено стање – процеси интрамолекуларних трансфера протона (ESIPT). Утицај везивања 3-HF и Dx70 на секундарну структуру BSA испитиван је преко циркуларног дихроизма. Испитиван је такође утицај темепературе (30–80 °C) на својствену флуоресценцију триптофана у систему 3-HF/BSA/Dx70. Резултати су дискутовани у односу на релевантност 3-HF, као осетљиве флуоресцентне пробе за испитивање интеракција флавон–протеин у плазми, као медијуму, као и у смислу биолошке евалуације.

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