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Binding of antioxidant flavone isovitexin to human serum albumin investigated by experimental and computational assays

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ABSTRACT

The flavonoids are a large class of polyphenolic compounds which occur naturally in plants where they are widely distributed. Isovitexin (ISO) is a glycosylated flavonoid that exhibits a potential antioxidant activity. Some recent studies have shown the pharmacokinetic activity of isovitexin in rat blood plasma, however, without detailing the molecular target that is linked and what physicochemical forces govern the interaction. In mammalians, the most abundant protein in blood plasma is the albumin and is not unlike with human, which human serum albumin (HSA) is the major extracellular protein and functions as a carrier of various drugs. The interaction between HSA and ISO was investigated using fluorescence, UV–vis absorbance, circular dichroism (CD), Fourier transform infrared spectroscopy (FT-IR) together with, computational methods like *ab initio* and molecular modeling calculation. Fluorescence quenching indicated that ISO location is within the hydrophobic pocket in subdomain IIA (site 1) of HSA, close to the Trp214 residue. The Stern–Volmer quenching constants determined at 288, 298 and 308 K and its dependence on temperature indicated that the quenching mechanism was static. From the analysis of binding equilibrium were determined; the binding site number and binding constants, with the correspondent thermodynamic parameters, ΔH, ΔG and ΔS for HSA–ISO complex. Also, a second binding analysis, binding density function (BDF) method, which is independent of any binding model pre-established obtained similar results. The fluorescence resonance energy transfer estimated the distance between the donor (HSA–Trp214) and acceptor (ISO), while FT-IR and CD spectroscopy measured possible changes of secondary structure at the formation of the HSA–ISO complex. The optimized geometry of isovitexin calculation performed with its ground state by using DFT/B3LYP/6-311+G(d,p) method. The HSA–ISO complex interactions determined by molecular modeling tool corroborated with the thermodynamic analysis from the experimental data.

1. Introduction

Flavonoids are a large class of naturally occurring polyphenolic compounds widely distributed in plants. The literature shows that flavonoids can present antioxidant, anticancer, antiviral, anti-inflammatory and heart disease protective activities [1]. Isovitexin (apigenin-6-C-β-D-glucopyranoside) is a glycoside flavonoid consisting of the flavone apigenin and the saccharide glucose, as shown in Fig. 1. Like many antioxidants present anti-inflammatory activity, ISO exhibits a potential antioxidant as well; studies have demonstrated that, in inflammatory processes induced by lipopolysaccharide (LPS) in mouse macrophage, this flavone is capable of inhibiting the production and, or release of tumor necrosis factor α (TNF-α) and prostaglandin E_2 (PG_2) [2]. Studies also have showed that isovitexin presents a possibly antiulcerogenic activity and it could help in peptic ulcer treatment [3]. ISO molecule is found, for example in rice hulls (*Oryza sativa* L.) [2], "*sempre-vivas chapadeira*" (*Syngonanthus bisulcatus* Rul.) [3] and tanxiang

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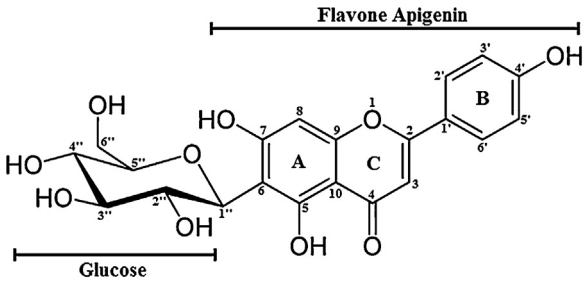

Fig. 1. (a) Chemical structure of isovitexin (apigenin-6-C-β-D-glucopyranoside).

(*Santalum album*) [4]. With such a promising profile as herbal medicine, pharmacokinetic studies were conducted in order to ascertain its presence in blood plasma after oral administration [4].

Human serum albumin (HSA) is the main extracellular protein, and it is present in high concentration, in blood plasma. HSA is a monomeric globular protein composed of three structurally similar domains (I, II and III), each containing two subdomains (A and B). Aromatic and heterocyclic ligands bind to HSA primarily within two hydrophobic pockets in subdomains IIA and IIIA, namely sites 1 and 2, respectively. Site 1 is the primary binding site for drugs like warfarin and phenylbutazone analogs, whereas diazepam and ibuprofen are bound primarily to site 2 [5]. The exceptional capacity of HSA to interact with several organic and inorganic molecules makes this protein an important regulator of intercellular fluxes and the main carrier for many drugs to different molecular targets. Therefore, more detailed studies of the microenvironment of binding sites of HSA are important for understanding the mechanism of HSA–drug interactions [5,6].

In this paper, a detailed study of the interaction between ISO and HSA was observed using spectroscopy methods including fluorescence spectroscopy, UV–vis absorbance, circular dichroism (CD), Fourier transform infrared spectroscopy (FT-IR), and computational methods like *ab initio* and molecular docking calculations. In particular, this work also made use of the binding density function (BDF) method for data analysis of fluorescence quenching that is very sparsely explored to study protein–ligand interaction. Such data set complements in great detail the information that is obtained in pharmacokinetic approaches of ISO, describing the non-covalent interactions that are responsible for the stability of the HSA–ISO complex.

2. Materials and methods

2.1. Materials and solutions

Human serum albumin fraction V was purchased from Sigma Chemical Co., and used as supplied. Isovitexin provided by Prof. Dr. Wagner Vilegas (UNESP, Brazil), which extraction and isolation were described previously [3]. All other chemicals were of analytical reagent grade and Milli-Q ultrapure water was used throughout the experiments. HSA was dissolved in aqueous and deuterated phosphate buffer solution of 50 mM at pH 7.0 (and pD 7.0) containing 0.15 M of NaCl. The stock solution of ISO was prepared in absolute ethanol. The aliquots of ISO applied in the following experiments were carefully evaluated to avoid aggregation of the flavonoid [7].

2.2. UV–vis absorbance spectroscopy

UV–vis absorption spectrum was recorded at room temperature on a Cary-3E spectrophotometer (Varian, Palo Alto, CA) equipped with 1.0 cm quartz cells of path length. UV–vis absorption spectra were recorded in the 250–500 nm range. Both concentrations of HSA and ISO were 4.0 μM. The final ethanol concentration in buffer was <1%.

2.3. Fluorescence spectroscopy

The fluorescence measurements were performed using an ISS PC1 steady-state spectrofluorimeter (Champaign, IL, USA) equipped with a Neslab RTE-221 thermostat bath. Both excitation and emission bandwidths were set at 8.0 nm. The excitation wavelength at 295 nm avoided the absorption of tyrosine residues, but excites the single tryptophan residue (Trp214) of HSA. The emission spectrum was collected in the range of 305–500 nm which was corrected for the background fluorescence of the buffer and for inner filter effects [8]. In the fluorescence quenching experiments, the titrations were performed by adding small aliquots from ISO stock solution to HSA solution (3.0 mL) at constant concentrations of 2.0, 4.0 and 8.0 μM. In experiments for Stern–Volmer and binding equilibria analysis, HSA concentration remained constant at 4.0 μM and ISO concentration varied from 0 to 4.8 μM with increment of 0.4 μM at 288, 298 and 308 K. For binding density function (BDF) method, the titration was performed at HSA constant concentrations of 2.0, 4.0 and 8.0 μM and ISO concentration varied from 0 to 3.4, 4.8 and 6.4 μM, respectively, at 298 K. The effect of ethanol as co-solvent was verified adding small aliquots to HSA solution (4.0 μM at 3.0 mL, 298 K) within the volume changes of the previous titrations. In all experiments, the final volume of ethanol in buffer was <1%.

2.4. Fourier transform infrared spectroscopy

The Fourier transform infrared (FT-IR) spectra obtained at room temperature utilized Nexus 670 FT-IR spectrometer (Nicolet, USA). All infrared spectra used the transmittance method in the range of 2200–1300 cm^{-1} with resolution of 4.0 cm^{-1} and 512 scans. The FT-IR experiments were performed with deuterated HSA solution of 20 mg/mL (0.3 mM) at pD 7.0. For the preparation of free ISO and HSA–ISO complex solutions, the solvent (ethanol) of ISO stock solution was evaporated and resuspended using the phosphate buffer and HSA solutions in D_2O, respectively, keeping the final concentration of ISO for the ligand–protein ratio of 0:1, 0.5:1 and 1:1. Subtraction, smoothing, baseline correction, second derivative, Fourier self-deconvolution and curve fitting are detailed in Supplementary material [9,10].

2.5. UV circular dichroism spectroscopy

All UV CD spectra recorded at room temperature at Jasco J-815 spectropolarimeter (Jasco, USA) and equipped with 0.01 cm quartz cell of path length. The range of UV CD spectra was from 190 to 260 nm with a scan rate of 20 nm/min, and total of 10 accumulations. The HSA concentration kept constant during all experiment at 4.0 μM while that the ISO to HSA ratios were of 0:1, 0.5:1 and 1:1. The final ethanol concentration in buffer was <1%. The equipment baseline correction and buffer spectrum was subtracted from free and complexed HSA. Secondary structures were estimated with CONTINLL software of CDPro package, using the reference set of proteins SMP56 [11].

2.6. Ab initio calculation

The Gaussian 09 program [12] provided by Núcleo de Computação Científica da Universidade Estadual Paulista (NCC/GridUNESP) was applied to the calculation of ISO structure. The optimized geometry calculated at the gas phase with ISO molecule isolated by using DFT/B3LYP/6-311+G(d,p) method. The next step, the vibrational frequency calculation, was performed to