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The interaction and binding of flavonoids to human serum albumin modify its conformation, stability and resistance against aggregation and oxidative injuries☆

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ABSTRACT

Background: Interactions of ligands with proteins imply changes in the properties of the macromolecules that may deeply modify their biological activities and conformations and allow them to acquire new and, sometimes, unexpected abilities. The flavonoid phloretin has several pharmacological properties that are starting to be elucidated, one of which is the well-known inhibition of glucose transport.
Methods: The interactions of phloretin to human serum albumin have been investigated by fluorescence, UV–visible, FTIR spectroscopy, native electrophoresis, protein ligand docking studies, fluorescence and scanning electron microscopy.
Results: Spectroscopic investigations suggest that the flavonoid binds to human serum albumin inducing a decrease in α-helix structures as shown by deconvolution of FTIR Amide I′ band. Fluorescence and displacement studies highlight modifications of environment around Trp214 with the primary binding site located in the Sudlow's site I. In the hydrophobic cavity of subdomain IIA, molecular modeling studies suggest that phloretin is in non-planar conformation and hydrogen-bonded with Ser202 and Ser454. These changes make HSA able to withstand protein degradation due to HCLO and fibrillation.
General significance: Our work aims to open new perspectives as far as the binding of flavonoids to HSA are concern and shows as the properties of both compounds can be remarkable modified after the complex formation, resulting, for instance, in a protein structure much more resistant to oxidation and fibrillation.
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1. Introduction

The binding of small molecules to proteins induces changes that deeply modify the binding, catalytic, thermodynamic and kinetic parameters of these macromolecules, often resulting in new and unexpected properties, such as the ability to counteract endogenous stresses and pathological conditions. Flavonoids are polyphenolic compounds with many interesting biological properties [1–10], which have been the focus of researches aimed at elucidating and discovering the molecular basis of their activities. Phloretin (Fig.1A), in particular, is a phenylpropanoid belonging to the dihydrochalcones flavonoid class, characterized by the lacking of a heterocyclic C-ring, and whose structure is closely related to those of their immediate precursors, the chalcones. Phloretin and its glycosylated forms are abundant in apple, kumquat and pear [11–13]. Moreover, phloretin glycosides have been found in sarsaparilla, sweet tea and in strawberry fruits [14–16]. Although the glycosylated forms are the most abundant natural source, they are nearly entirely converted into phloretin by hydrolytic enzymes in the small intestine and introduced in the blood circle [17]. Phloretin displays a broad spectrum of bioactivities such as anticancer, antibacterial, antiviral, anti-inflammatory properties and it is one of the naturally occurring non-steroid estrogens [13,18–22]. It has strong antioxidant activity due to its 2,6-dihydroxyacetophenone group [23,24] and it has been known for a long time to be a competitive inhibitor of sodium-glucose cotransporter [25]. Phloretin interacts with biological membranes increasing the fluidity of the intracellular lipid bilayers, making it suitable, from the pharmacological point of view, as a penetration

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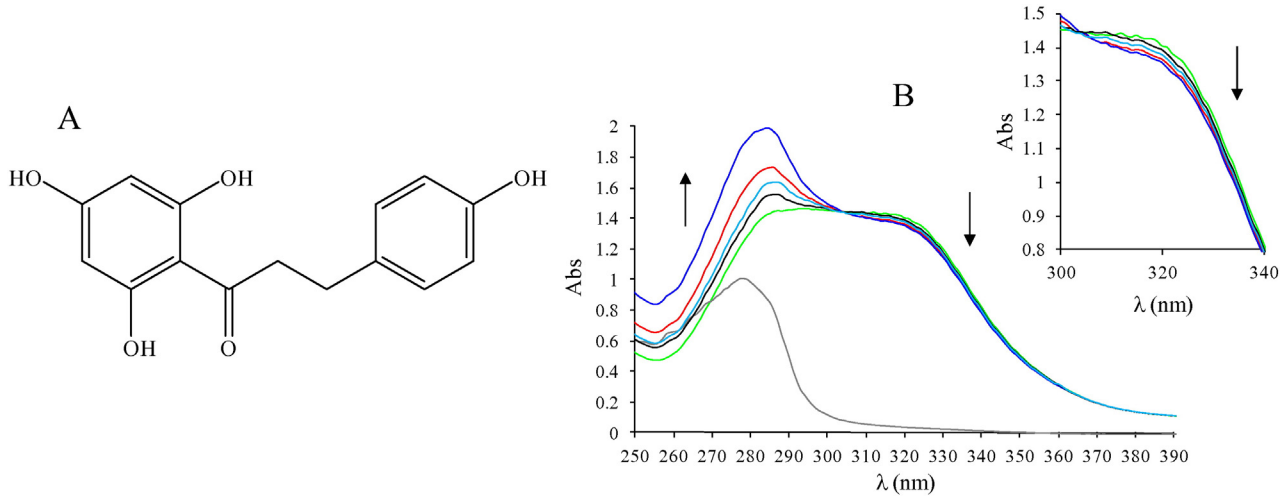

Fig. 1. Chemical structure of phloretin (A) and UV–visible absorption spectra (B) of phloretin (60 μM) in the absence (green line) or in the presence of HSA 15 (black line), 25 (cyan line), 30 (red line) and 60 μM (blue line) at pH 7.4. In the graph is also depicted the generic spectrum (gray line) of only protein (30 μM). The inset depicts a magnification of the band at 320 nm.

enhancer to extend the availability of drugs administered, for instance, via the skin trans-membrane transport [26]. Finally, it inhibits the formation of AGEs and down-regulates the secretion of IL-2 of human normal lymphocytes [27–29].

Human serum albumin (HSA) has countless functions: it is one of the main responsible of oncotic plasma pressure and the most important carrier of endogenous and exogenous compounds present in human body [30]. Its role in the blood substances transportation is so much important that the bioavailability of many biologically active compounds is related to the interaction and successive binding with HSA. The binding of substances to macromolecules induces oftentimes modifications of weak interactions, resulting in changes of their structure and stability [31–36]. HSA is a globular protein composed of 585 amino acids with a molecular weight of 66,500 Da, characterized by the presence of α-helices structures [37]. Three homologous domains (domains I–III), in turn divided into 2 subdomains (A and B), with multiple ligand-binding sites localized in each of these subdomains have been described [37–39]. In this paper, the interaction between phloretin and HSA has been carried out by means of multi-spectroscopic methods and docking studies, obtaining important information such as association and change of protein secondary structure, to analyze, in depth, the potentiality of the formed complex to decrease/avoid oxidative stress and protein fibrillation.

2. Materials and methods

2.1. Materials

HSA and phloretin were supplied by Sigma-Aldrich and used without further purification, to be closer to *in vivo* conditions. All the other reagents were of analytical degree.

2.2. UV–visible spectroscopy spectra

Samples for UV–visible spectroscopy in buffer solutions (pH 7.4 or 7.7) were prepared by adding an aliquot of a phloretin solution in dimethyl sulfoxide (DMSO) up to the final desired concentration. Interactions of the ligand with albumin have been studied by UV–Visible spectroscopy adding to 60 μM phloretin solution increasing amount of HSA up to 60 μM. UV–Visible absorption spectra were recorded from 250 to 390 nm utilizing a spectrophotometer with 1.0 cm optical path quartz cuvette. Spectra of buffer solutions were collected at the same condition and subtracted from those of sample solutions.

2.3. Fluorescence spectra

Fluorescence spectra were recorded using a FluoroMax-4 spectrofluorometer by Horiba Jobin-Yvon equipped with a pulsed xenon lamp and an F-3006 Autotitration Injector with two Hamilton Syringes (mods. Gastight 1725 and 1001 TLLX, 250 μL and 1.0 mL capacity, respectively). The resolutions of wavelength selectors and titrant additions were 0.3 nm and 0.25 μL, respectively. The instrument was also equipped with a Peltier Sample Cooler (mod. F-3004) controlled by a Peltier Thermoelectric Temperature Controller model LFI-3751 (5 A — 40 W). The whole system was controlled by the FluorEssence 2.1 software by Horiba Jobin-Yvon. The titrations were performed directly in a Hellma type 101-OS precision cell (Light Path 10 mm), where a magnetic stirrer and the anti-diffusion burette tip were placed in a position that would not interfere with the light beam. The automatic data acquisition (fluorescence intensity *vs.* λ (nm) for each titrant addition), was performed using the same FluorEssence 2.1 software. The excitation and emission bandwidths were both 5 nm. The protein samples were excited at 280 and 295 nm to characterize a possible different behavior of tryptophan and/or tyrosine residues. It was observed that both spectra were similar. The rest of the experiment was acquired by excitation at 280 nm and emission spectra were recorded in the range of 300–400 nm at 25, 31 and 37 °C. The potential interaction between phloretin and HSA was performed by fluorimetric titration. A 2.0 mL solution containing 1.5×10^{-5} mol/L HSA in 20 mM sodium phosphate buffer (pH 7.4) was titrated by successive additions of phloretin stock solution (1.0×10^{-3} mol/L) to give a final concentration ranging from 0 to 1.5×10^{-5} mol/L. The displacement studies were carried out utilizing different site markers (warfarin, ibuprofen, and digitoxin for sites I, II, and III, respectively). A solution of HSA (1.5×10^{-5} mol/L) in 20 mM sodium phosphate buffer (pH 7.4) containing phloretin at a the same final concentration (1.5×10^{-5} mol/L) was titrated by successive additions of site markers solution (1.0×10^{-3} mol/L) to obtain an overall site marker concentration ranging from 0 to 9×10^{-5} mol/L. Fluorescence spectra were recorded at 37 °C as above reported. The fluorescence of the ternary mixture as a percentage of the initial fluorescence was determined according to the method of Sudlow et al. [39]:

$$\frac{F_2}{F_1} \times 100$$