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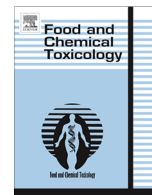

The binding affinity of phthalate plasticizers-protein revealed by spectroscopic techniques and molecular modeling

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A B S T R A C T

Phthalate plasticizers have been subjected to close scrutiny and evidences of their toxicity and other negative environmental impacts have arisen as a result of their use in food in some countries. Once entering human body, plasticizers could affect the conformation of human serum albumin and protein function. The interaction between two phthalate plasticizers and human serum albumin was investigated by multispectroscopic techniques and molecular modeling. The alteration in protein conformational stability was determined by fluorescence quenching data. The thermodynamic parameters indicated that the hydrophobic interactions played a major role in the process. In addition, the alterations of HSA secondary structure in the presence of phthalate plasticizers were investigated. Molecular modeling and displacement experiments showed that phthalate plasticizers situated within subdomain IIA (site I) of HSA. Furthermore, the binding distances for the plasticizers–HSA system were provided by the efficiency of fluorescence resonance energy transfer.

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1. Introduction

Nowadays, the toxicity of phthalate plasticizers is of considerable interest because of their use in many consumer products leading to widespread human exposures and environmental contamination (Wittassek et al., 2011). Phthalate plasticizers are manufactured world-wide on a large scale, being mainly produced for use as plasticizers of common packaging materials such as plastics, rubber, and cellulose, especially as a softener in Polyvinyl chloride (PVC). Extensive application of phthalate plasticizers has resulted in their ubiquitous presence and the accumulation in the environment (Guo and Kannan, 2012). Human exposure to these materials is inevitable as they can enter the body via all kinds of paths, accumulate in the bodies and cause a deleterious effect.

Recently, new information regarding the presence of Di-isodecyl phthalate (DIDP) in Augmentin oral suspensions was reported by the media in Taiwan. This scandal has caused shock and panic among the majority of Taiwanese people and has attracted international attention (Kamrin, 2009). DIDP belongs to phthalates which is used as plasticizer. Many studies have suggested associations between DIDP exposure and increased sperm DNA damage, decreased proportion of sperm with normal morphology, decreased sperm concentration, decreased sperm morphology, sex hormone alteration (Yen et al., 2011). Simultaneously, another phthalate plasticizer Di-*n*-octyl phthalate (DNOP) added to LP33 capsule was exposed by Taiwanese media. Harmful effects in animals such as liver toxicity have mainly been reported with high amounts of DNOP (Sarkar et al., 2013). Therefore, DNOP was listed as the first class of poison by Taiwan's Environmental Protection Administration (EPA). The ubiquitous use of phthalate plasticizers results in environmental pollution and be directly taken up by animals *via* food or air and potential bioaccumulation (Casajuana and Lacorte, 2004). Apart from its severe impact on environment, the phthalate plasticizers have properties that make it a potential risk to human health of considerable significance. This inspires us to study mechanisms of phthalate plasticizers (DNOP and DIDP, structures shown in Fig. 1) and human serum albumin (HSA).

HSA is the most abundant protein in the bloodstream at concentrations of 50 mg/mL. HSA helps to maintain the osmotic pressure of the body and is an important carrier of fatty acids, metabolites, drugs and other important ligands (Oratz et al., 1988; Carter and Ho, 1994; Dong et al., 2014; Rozga and Bal, 2010). It consists of a single polypeptide chain made up of 585 amino acid residues. At biological pH the HSA chain forms three homologous domains (I–III), each of which is divided into two subdomains, termed A and B, having six and four α-helices, respectively. HSA accounts

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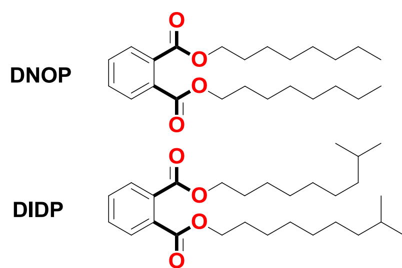

Fig. 1. The chemical structures of DNOP and DIDP.

for the binding and transportation of a large number of small molecules including exogenous as well as endogenous compounds. The distribution and metabolic lifetime of these compounds in the body as well as biological activity largely depend on their affinity to HSA. Consequently, the investigation of the interaction between phthalate plasticizers and HSA has great significance. To the best of our knowledge, the mechanisms that caused conformational changes in HSA by the DNOP/DIDP have not been reported.

In this work, a comprehensive investigation was performed for the binding properties of phthalate plasticizers to HSA under the physiological conditions. The conformation of HSA and the binding mechanism of above phthalate plasticizers with HSA were investigated by optical spectroscopic techniques (UV–vis absorption, steady-state, three-dimensional and time-resolved fluorescence, FT-IR). Molecular docking was employed to gain insight into the binding modes and further identify the key residues for binding. In addition, fluorescence displacement experiments of the two plasticizers to HSA were investigated. The findings of this study can provide meaningful clues for clarifying the binding mechanism of plasticizers with HSA and is helpful for understanding its effect on protein function in the sight of the food security.

2. Materials and methods

2.1. Materials

HSA (fatty acid free <0.05%), purchased from Sigma–Aldrich (St. Louis, MO, USA), was used without further purification. Final concentrations of the solutes were verified spectrophotometrically using the molar absorption coefficients determined in this work: ε_{280} = 35,500 M^{-1} cm^{-1} for HSA (Manzini et al., 1979). DIDP, DNOP, phenybutazon (PB), and flufenamic acid (FA) were of analytical grade, and purchased from J&K Scientific Ltd. (Beijing, China). Other reagents such as NaCl, Tris were all of analytical grade. Stock solutions of HSA (3.0×10^{-5} M) was prepared in Tris–HCl buffer (0.10 M) of pH 7.4. The stock solutions of 1.0×10^{-3} M DIDP and 1.0×10^{-3} M DNOP were prepared in anhydrous methanol, and 1.0×10^{-3} M PB, 1.0×10^{-3} M FA 1.0×10^{-3} M Dig were prepared in anhydrous methanol. Ultrapure water from a Milli-Q ultrapure water purification system was used throughout the experiments. All pH values were measured with a pH-3 digital pH meter (Shanghai Lei Ci Device Works, Shanghai, China) with a combined glass electrode, which was calibrated with standard pH buffer solutions. Thermo/HAAKE DC30-K20 refrigerated circulator bath (±0.01 °C accuracy) was used to control the temperature of the samples.

2.2. Apparatus and methods

Fluorescence spectra were recorded with a FP-6500 spectrofluorometer (JASCO, Japan. The bandwidths used for the excitation and the emission were 5 nm. Fluorescence spectra were corrected for the background intensities of the buffer solution. Each fluorescence spectrum of the protein in presence of different ligand concentrations were corrected for any possible inner filter effect using the following equation (Lakowicz, 2006):

$$I_F^{corr}(\lambda_E, \lambda_F) = I_F(\lambda_E, \lambda_F)\frac{A(\lambda_E)}{A_{tot}(\lambda_E)} \quad (1)$$

where A represents the absorbance of the free protein, and A_{tot} is the total absorbance of the solution at the excitation wavelength (λ_E). The intensity of fluorescence used in this paper is the corrected fluorescence intensity.

Synchronous fluorescence spectra of HSA in the absence and presence of increasing amounts of phthalate plasticizers were measured under the same conditions with steady-state fluorescence. The $\Delta\lambda$ between the excitation and emission wavelengths was set at 60 nm for studying the characteristics of intrinsic amino residue tryptophan (Trp).

Fluorescence titration experiments: HSA (3.0×10^{-6} mol/L) solution was titrated manually by successive addition DNOP and DIDP (1.0×10^{-3} mol/L), respectively. The fluorescence intensity was subsequently measured and the fluorescence intensity was measured after 3 min (excitation at 280 nm and emission at 332 nm) at four different temperatures ((289, 296, 303, and 310) K). The temperature was kept by circulating water throughout the experiment.

Three-dimensional fluorescence spectra were obtained under the following conditions: the emission wavelength was recorded between 220 and 600 nm, the initial excitation wavelength was set to 220 nm with increment of 5 nm, and the other scanning parameters were identical to those used for steady state fluorescence as mentioned above.

Fluorescence lifetime measurements were obtained with a FL 980 spectrofluorimeter (Edinburgh Instruments) using time correlated single photon counting method. Excitation was performed at 295 nm with a nanoLED. Data analysis was performed with the non-linear least-square deconvolution software provided with the instrument. In both time-resolved techniques the instrumental response function (IRF) was determined experimentally based on the light signal scattered from Ludox (colloidal silica in water), and used for subsequent deconvolution of the fluorescence signal. Average fluorescence lifetime (τ) for triexponential iterative fittings was calculated from the decay times and the normalized pre-exponential factors using the following equation:

$$\langle\tau\rangle = \frac{\sum \alpha_i \tau^2}{\sum \alpha_i \tau} \quad (2)$$

where α_i are the pre-exponential factors and τ are the respective decay times.

UV–vis spectroscopy was performed on a UV-1700 PharmaSpec (Shimadzu, Japan) room temperature from 250 to 350 nm using a quartz cuvette with 1 cm path length.

FT-IR spectra were recorded using a Tensor 27 FT-IR spectrometer (Bruker, German) with a Germanium attenuated total reflection (ATR) accessory. All spectra were taken *via* the ATR method with a resolution of 4 cm^{-1} using 60 scans. Spectra processing procedures involved spectra of buffer being collected under the same conditions. The FT-IR spectra of HSA in presence and absence of sample were recorded. First, the buffer solution spectra were collected. Next, the free HSA spectra were acquired by subtracting the absorption of the buffer solution from the absorption spectrum of HSA. Finally, the HSA difference spectrum was obtained by subtracting the sample-free spectrum from the sample-HSA spectrum. For the subtraction criterion, the original spectrum of protein solution between 2200 cm^{-1} and 1800 cm^{-1} is a smooth straight (Dong et al., 1990). The Fourier self deconvolution and second derivative were applied respectively to estimate the number, position and width of the component bands. Based on these parameters, curve-fitting process was carried out to obtain the best-fit Gaussian-shaped curves to the original protein spectrum. All assays were executed in triplicate, and all statistic data were treated using the OriginPro Software (OriginLab Corporation, USA).

Before displacement, PB (3.0 μM), FA (3.0 μM) and Dig (3.0 μM) were incubated with HSA (3.0 μM), pH 7.4, at 298 K for 30 min. Then a 3.0 mL sample was added to a 1 cm quartz cuvette, followed by titration of sample. The emission intensity of HSA was observed from 290 to 500 nm at a λ_{ex} of 280 nm.

The docking experiments were achieved using the docking software AutoDock 4.2 and AutoDock Tools (ADT) to identify the potential ligand binding sites (Lv et al., 2013). The crystal structure of HSA was obtained from the Protein Data Bank (PDB code 1H9Z). All water molecules were removed and the polar hydrogen and the Gasteiger charges were added at the beginning of docking study. The grid box size of HSA-phthalate plasticizers systems were 60 Å × 60 Å × 60 Å (grid spacing of 0.375 Å), which encompassed the whole subdomain IIA. On the basis of the Lamarckian genetic algorithm (LGA), 100 runs were performed for ligands with 150 individuals in the population; the maximum number of energy evaluation was set at 2,500,000 and others used were default parameters. In the docking run the root mean square cluster tolerance was set to 2.0 Å and were ranked by binding energy values.

3. Result and discussion

3.1. Fluorescence quenching

The application of fluorescence measurements can reveal the reactivity of chemical and biological systems since it allows non-intrusive measurements of substances in low concentration under physiological conditions. Intrinsic fluorescence of HSA originates from tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) residues. The intrinsic fluorescence of HSA is very sensitive to its microenvironment and almost contributed by Trp alone, because

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