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Elucidation of energetics and mode of recognition of green tea polyphenols by human serum albumin

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ABSTRACT

Green tea is rich in several polyphenols which has many beneficial biological effects. We have done a detailed qualitative and quantitative study on binding of two polyphenol (−)-epicatechin (EC) and (−)-epigallocatechin-3-gallate (EGCG) with human serum albumin (HSA) by using a combination of isothermal titration calorimetry (ITC), differential scanning calorimetry (DSC), steady-state fluorescence, and circular dichroism spectroscopies. The binding constants obtained from isothermal titration calorimetry for EC-HSA and EGCG-HSA complexes are $(2.09 \pm 0.08) \times 10^4$ and $(1.84 \pm 0.30) \times 10^5$, respectively. The effects of ionic strength and additives of polar and non-polar characters indicate contribution of a mix of ionic, hydrogen bonding and hydrophobic interactions in the polyphenol-HSA association process. The 3D fluorescence analysis and circular dichroism (CD) studies reveal that the complexation of EGCG induces significant conformational change in HSA. The DSC results also show that the binding of EGCG is stronger compared to that of EC and provides quantitative information on the effect of these polyphenols on the thermal stability of HSA. Such studies play a crucial role in understanding the mechanism of drug-protein interactions and optimization of various binding parameters for successful drug delivery.

1. Introduction

Human beings are frequently exposed to adverse effects of various environmental pollutants including free radicals. These environmental pollutants and free radicals induce oxidative stress in the organism, also known as environmental stress [1]. Oxidative stress is known to be a key factor in the pathogenesis of various human diseases and is also responsible for physiological process of aging [2]. Consequently, significant effects have been made to study the effect of naturally occurring ingredients having antioxidant activity, which could protect human beings against environmental pollutants and free radicals [3]. Green tea contains various types of flavonoids which are mainly polyphenols such as (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin-3-gallate (EGCG) and elagic acid (EA). These polyphenols possess antioxidant as well as anticancer properties, and hence considered as important chemopreventive natural products [4,5]. Epicatechin (EC) (Fig. 1A) has been found to improve cerebral blood flow, synaptic plasticity, and mitochondrial function, hence reduces age-related neurological deterioration and the risk of neurodegenerative diseases [6]. EC also protects neurons from oxidative damage by suppressing c-Jun N-terminal kinases [7,8] and prevents NMDA induced stroke damage in mice [9]. EC is loaded with anti-oxidative properties and also mediates beneficial vascular functions in humans [10,11]. EC prevents β-amyloid ($A\beta_{25-35}$)-induced apoptosis in PC12 cells [12], and reduces hippocampal toxicity caused by $A\beta_{25-35}$ in rats [13].

Another polyphenolic flavonoid EGCG (Fig. 1B) is a galloylated catechine and is known to be the most abundant and powerful antioxidant present in green tea for cancer chemoprevention [14]. EGCG works synergistically with anticancer drugs for cancer chemoprevention and inhibits colon cancer cell growth [15]. EGCG is reported to show powerful antiproliferative effects and significantly induces cell cycle arrest in the G1 phase and causes cell apoptosis in human colorectal cancer cell lines, HCT-116 and SW-480 [16]. EGCG directly interacts with proteins and phospholipids present in the plasma membrane. It regulates specific intracellular signal transduction pathways, mitochondrial function as well as transcription and exerts various favourable biological activities [17–20]. A thorough literature search suggests that EGCG also binds to the T-cell receptor, CD4 with high affinity and can be a potential molecule for HIV-1 therapy [21]. Some latest studies also indicate that binding of green tea polyphenols with blood plasma proteins modulate their plasma concentration and biological activities [22].

Human serum albumin (HSA) is a highly soluble negatively charged protein and is the major drug binding component present in blood [23]. HSA offers multiple binding sites for binding a wide range of

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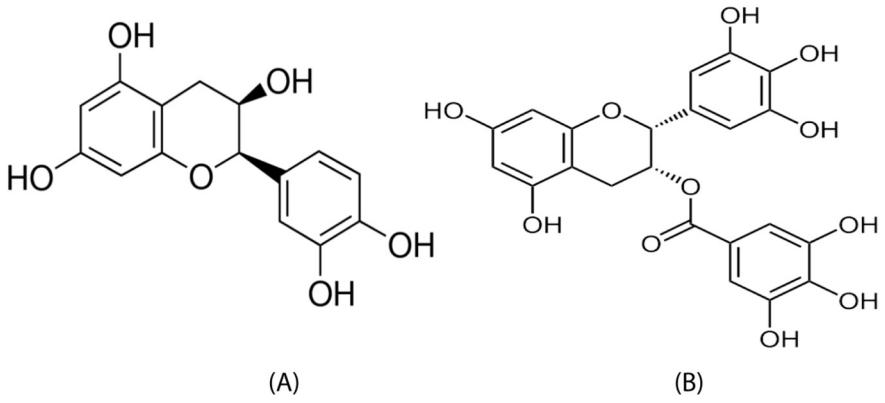

Fig. 1. Molecular structure of (A) EG and (B) EGCG.

endogenous and exogenous ligands. Serum albumin-drug binding is considered to have a significant impact in the rate of diffusion of drug between plasma and tissues and hence influences absorption, distribution and elimination of drugs [24]. HSA contains three homologous domains (I, II, and III) which are further divided into a pair of subdomains termed subdomain-A and subdomain-B. HSA has two major binding-sites for drugs which are sometimes also referred to as warfarin-azapropazone site or Sudlow site I, and indole-benzodiazepine site or Sudlow site II [25]. In addition, a third binding pocket is also present within subdomain-IB referred as bilirubin or hemin site [26].

Although a detailed literature search shows various studies for the interactions of different polyphenols with serum albumin [27–31], a detailed thermodynamic investigation for interactions of flavonoids EG and EGCG with the carrier protein is missing. In the present work we have done a detailed thermodynamic investigation of interactions of EC and EGCG with HSA using ultrasensitive isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC). In addition to that we have also used various spectroscopic techniques such as fluorescence spectroscopy and circular dichroism spectroscopy (CD) for detailed conformational characterization of HSA upon binding with EC and EGCG. This study is very important not only to provide a better understanding about the prospect of transportation of EC and EGCG through the Serum albumin but also to optimize various binding parameters for successful drug delivery.

2. Experimental methodology

2.1. Materials

Human serum albumin (HSA, ≥ 0.97), epicatechin (EC, ≥ 0.90), epigallocatechin gallate (EGCG, ≥ 0.95), sodium chloride (≥0.99), sucrose (≥0.99), tetrabutylammonium bromide (≥0.98), sodium dodecyl sulphate (SDS, 0.98), hexadecyltrimethylammonium bromide (HTAB, ≥0.98), sodium phosphate (≥0.96), warfarin (1.00) and Hemin (≥0.90) of the best available purity grade were purchased from Sigma Aldrich Chemical Company USA. The numbers given in the parenthesis are the mole fraction purities as reported by the suppliers. MilliQ water from Merk Millipore was used to prepare all solutions. All the masses were determined with a Sartorius BSA224S-CW digital balance which has a readability of 0.1 mg. The solutions were prepared in 20 mM phosphate buffer at pH = 7.4 which was measured on a Lab India PICO$^+$ pH-meter at ambient temperature. The stock solutions of HSA was prepared by extensive overnight dialysis against the buffer at $T = 4$ °C with at least three changes of buffer. The final dialysate buffer was used to prepare all other solutions used in the experiments. The concentration of human serum albumin was determined on a UV-1800 Shimadzu UV–visible spectrophotometer at $\lambda = 280$ nm using molar extinction coefficient of 35,700 M^{-1} cm^{-1} [32].

2.2. Isothermal titration calorimetry

The thermodynamic parameters for the interaction of polyphenols with HSA were determined using an isothermal titration calorimeter (MicroCal iTC200, Malvern, UK). The EC/EGCG solution taken in the syringe was titrated with HSA contained in the sample cell using a 40 μl rotating stirrer-syringe, and the reference cell contained the same buffer. Each experiment consisted of 19 consecutive injections of 2 μl of 1.875 mM EC/EGCG solution into 75 μM HSA solution. The titration curves for the ligand dilutions and protein dilutions were subtracted from the respective heat of binding reaction of HSA-EG/EGCG to obtain the effective heat of interaction. The time interval between the consecutive injections was fixed at 120 s and each injection had duration of 4 s. The resulting titration heat profiles were fitted well to single-site binding model using Origin 7.0 software, provided by Microcal iTC200. In this manner the values of binding constant (K), standard molar enthalpy change (ΔH_m^0), standard molar entropy change (ΔS_m^0) and stoichiometry (n) was obtained. In order to obtain the values of standard molar Gibbs free energy change (ΔG_m^0), standard thermodynamics equation (Eq. (1)) was used.

$$\Delta G_m^0 = -RT\ \ln K \tag{1}$$

All experiments were done at least in triplet to ensure reproducibility of the data.

2.3. Fluorescence spectroscopy

Fluorescence spectra were recorded on an Agilent spectrofluorimeter using an excitation wavelength of 295 nm. The excitation and emission slit widths were fixed at 5 nm each. For the selective excitation of tryptophan residues, the excitation wavelength was set at 295 nm.

Quantitative investigation of the binding of HSA and EG/EGCG was done by performing fluorimetric titration by successive addition of the polyphenols. Here, the protein concentration used was 15 μM and the experiments were performed at five different temperatures (15 °C, 20 °C, 25 °C, 30 °C and 37 °C). Blank spectra containing the same amount of ligand as in the sample were collected and subtracted from that of the complex. The 3-D fluorescence spectra of unbound HSA (15 μM) and HSA-EC/EGCG complex (molar ratio, 1:20) were recorded in an excitation wavelength range of 200–350 nm with an increment of 5 nm. The emission wavelength range was fixed as 200–500 nm.

2.4. Circular dichroism measurements

The far-UV (190–250 nm) and near-UV (250–350 nm) CD spectra of HSA at increasing concentration of EC/EGCG were recorded on a JASCO-J815 spectropolarimeter in order to observe the alterations in the secondary and tertiary structures of the protein. The concentration of