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Deciphering the inhibitory mechanism of genistein on xanthine oxidase *in vitro*

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ABSTRACT

Genistein (Gen), widely distributed in soybean, is proved to be important in homeostasis in the human body. Herein, the inhibitory mechanism of Gen against xanthine oxidase (XO) was studied through multispectroscopic methods and molecular simulation. The inhibition kinetics showed that Gen competitively inhibited XO with an inhibition constant of (1.39 ± 0.11) μM by competing with xanthine for binding to the active site of XO. Fluorescence titration study suggested that the fluorescence quenching mechanism of XO was static, resulting from the formation of a Gen–XO complex at one fold site. The calculated thermodynamic parameters revealed that the interaction process was driven mainly by hydrophobic interactions and hydrogen bonds with affinity of (5.24 ± 0.02) × 10^4 L mol^{-1}. Conformational analyses demonstrated that the microenvironment and the secondary structure of XO were changed upon binding of Gen. The molecular docking displayed that Gen bound to the active cavity of XO by interacting with the surrounding amino acid residues (Leu648, Phe649, Glu802, Ser876, Glu879, Arg880, Phe914, Phe1009, Thr1010 and Phe1013). Thus, the inhibition may be attributed to the insertion of Gen into the active site of XO occupying the catalytic center of the enzyme to avoid entry of the substrate and inducing conformational changes of XO (more compact), which was further unfavorable for forming the active cavity and further reduced the landing and oxidation of substrate. This study may offer novel insights into the inhibition mechanism of Gen on XO.

1. Introduction

Xanthine oxidase (XO) is a molybdenum (Mo)-containing enzyme which belongs to the Mo hydroxylase family of protein. XO controls the sequential hydroxylation of hypoxanthine to xanthine then to uric acid, accompanying with the generation of reactive oxygen species (ROS), as depicted in Scheme 1. The Mo center is responsible for the oxidative hydroxylation of substrates, which results in the two-electron reduction of the Mo center from Mo (VI) to Mo (IV). Following the reduction of the Mo, electrons are passed *via* two [2Fe–2S] clusters to a FAD cofactor [1,2]. However, the over-activity of XO within the living system is known to be associated with the excess levels of blood uric acid (hyperuricemia), which is the underlying cause of gout. Thus, a specific inhibitor for XO may be a significant therapy of hyperuricemia, gout and some other related diseases induced by the concomitant subversive free radicals including DNA damage [3], ischemia-reperfusion injury, heart attacks, stroke and renal hypoxia [4,5]. Side effects of the main XO inhibitor allopurinol in clinic including bone marrow depression, hepatotoxicity, and Stevens Jones syndrome, collectively known as allopurinol hypersensitivity syndrome, have been reported, especially in patients with renal insufficiency [1,6]. Besides allopurinol, several synthesized purine analogs such as 1-acetyl-3,5-diaryl-4,5-dihydro (1H) pyrazoles [7], N-(1,3-Diaryl-3-oxopropyl)amides [8], aza-flavones [9], naphthopyrans [10] were developed as potent XO inhibitors. Singh et al. [11] reported that NF-4 with p-fluoro phenyl ring was a potent inhibitor with IC_{50} value of 0.62 μM and found that the naphthoflavones displayed mixed type inhibition.

Comparing with those synthesized inhibitors, the researchers also attempted to discover new natural inhibitors with low side effects, especially the "multifunctional inhibitors" with diverse efficacy in recent years. Previous studies have clearly shown that some flavonoids exhibited inhibition on XO activity [12–14]. As a kind of natural inhibitors in fruits, vegetables, tea and other natural products with a variety of biological activities and low toxicity, flavonoids have attracted increasing attention. For example, apigenin as a kind of common flavonoids possessed a strong inhibition activity against XO with respect to xanthine with an inhibition constant (K_i) value of (0.61 ± 0.31) μM [14]; Luteolin has been found to inhibit XO activity with K_i of (1.9 ± 0.7) μM [13]. Among which, isoflavonoids exhibited a variety of additional biological activities such as anticancer, antitumor and antimetastatic properties [15]. Especially, it is a very strong antioxidant that removes oxygen free radicals in the body effectively. One of the major isoflavonoids in soy, genistein (4′,5,7-Trihydroxyisoflavone, Gen) has

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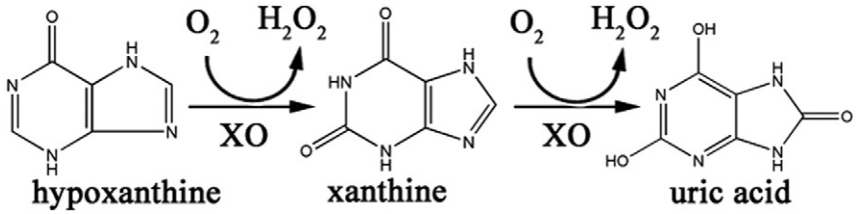

Scheme 1. Schematic diagram of the purine degradation pathway with the catalysis of XO.

numerous biological activities including regulation of estrogen-mediated events, modulation of apoptosis and inhibition of DNA topoisomerase activities [16]. Meanwhile, there were studies on Gen as an enzyme inhibitor. Akiyama et al. found that Gen acted as a specific inhibitor of tyrosine-specific protein kinases [17]; Markovits et al. have reported that Gen was an inhibitor of both protein tyrosine kinases and mammalian DNA topoisomerase II [18]; Lee et al. investigated the inhibition of Gen on the activity of α-glucosidase [19]. In addition, Hayashi [20] reported inhibition of XO by Gen, which was then proved by Van Hoorn [21]. Recently, Lin et al. [22] have compared the inhibitory properties towards XO of some flavonoids, demonstrating that Gen inhibited XO with a low IC_{50} (4.83 μM). To our knowledge, little information on the inhibitory mechanism of Gen against XO was available.

Thus, this work was designed to investigate the inhibitory mechanism of Gen against XO. A computer simulation was applied to predict the binding of Gen into the active site of XO. Then, the interaction between Gen and XO was studied *in vitro* by using multispectroscopic methods including UV–vis absorption, fluorescence, Fourier transform infrared (FT-IR) and circular dichroism (CD) spectroscopy. The result of enzymatic kinetic analysis of Gen towards XO proved to be a reversible and competitive inhibition. Meanwhile, the quenching mechanism, binding constant, the number of binding sites, thermodynamic parameters and the conformational change of XO induced by Gen were investigated in detail. Generally, the results cannot only give us a comprehension of the behavior of Gen as an XO inhibitor, but also provide useful guidance for the design of effective enzyme inhibitors. On the other hand, clarifying the inhibition mechanism may provide new insights into the application of the nutrimental dietary sustenance which would be beneficial for patients with gout.

2. Experimental

2.1. Chemicals and Materials

XO (from bovine milk, 35.7 units mL^{-1}), xanthine (152.11 g/mol) and allopurinol (136.11 g/mol) were all purchased from Sigma-Aldrich Corp (St. Louis, USA) and then prepared as stock solutions with Tris–HCl buffer (50 mM, pH 7.4). Their stock solutions were both freshly prepared just before the experiments. Gen (98%, 270.24 g/mol) obtained from Aladdin Chemistry Co. Ltd. (Shanghai, China) was dissolved in absolute ethyl alcohol as a stock solution. To avoid the effect on the enzyme structure and activity, the final alcoholic concentration in the experiments was less than 1% (assured by experiments). All stock solutions were stored at 0–4 °C, and all other reagents were of analytical reagent grade. The freshly deionized water (18.2 MΩ cm) was produced by Millipore Simplicity Water Purification System (Millipore, Molsheim, France). The pH of Tris–HCl buffer was measured to be 7.4 by a digital pH-meter with a glass-calomel electrode.

2.2. Methods

2.2.1. Molecular Modeling study

XO from bovine milk used in this study was obtained from PDB data base (ID: 3ETR). The X-ray crystal structure was in complex with lumazine (LUZ, the substrate that connects with Mo). XO is a homodimer with each monomer independently possessing one Mo center, two [2Fe–2S] clusters and one FAD cofactor. The four redox-active centers are found in separate domains of the polypeptide, and the Mo center is in Chain C [23]. The structure of Gen was extracted from Chem3D Ultra 8.0. The molecular simulation analysis of Gen to XO was performed by AUTODOCK 4.2 (Scripps, USA) at suppositional temperature of 298 K. A dimension grid box (110 Å × 100 Å × 110 Å) was defined to enclose the active site with a certain grid spacing (0.729 Å). Before assigning Gasteiger charges to the macromolecule file, all the water molecules were removed and the hydrogen atoms were added. The remaining genetic algorithm parameters were maintained at their default values. In order to validate the crystal structure of the ligand, the docking was carried for 100 genetic algorithm runs, which was considered as the optimum number required [24]. The best docking result was considered as the one with the lowest Δ*G* (estimated binding energy) calculated by the following equation:

$$\begin{aligned}\Delta G\ (\text{estimated binding energy}) = {} & \Delta G\ (\text{intermolecular energy}) \\ & + \Delta G\ (\text{torsional energy}) \\ & + \Delta G\ (\text{internal energy}) \\ & - \Delta G\ (\text{unbound extended energy})\end{aligned} \quad (1)$$

where Δ*G* (intermolecular energy) denotes the sum of the Van der Waals, hydrogen bond, electrostatic, and desolvation energies; Δ*G* (torsional energy) is torsional free energy; Δ*G* (internal energy) represents the final total internal energy and Δ*G* (unbound extended energy) shows the energy of unbound system. The unit of the energy is kcal mol^{-1} [24].

2.2.2. Enzyme Activity Assay

The inhibitory activity of Gen against XO was carried out on Shimadzu UV-2450 spectrophotometer (Shimadzu, Japan) with a 1.0 cm quartz cell by using a method previously reported [23]. In brief, an array of mixtures containing XO (final concentration was 75 nM), different concentrations of Gen and Tris–HCl buffer were kept in the volume of 2 mL. Then the mixtures were incubated at 37 °C for 3 h. The reaction was started by adding substrate xanthine (final concentration was 50 μM), and the absorbance of the solution was measured at 290 nm within 1 min. Allopurinol was used as a positive control. The relative activity of XO was calculated as follows: relative activity of XO (%) = $(A/B) \times 100$, where *A* is the reaction rate of system containing test sample; *B* is the reaction rate of system without test sample. Half maximal inhibitory concentration of a substance (IC_{50}) value was calculated from the mean data of triplicate experiments.

2.2.3. Kinetic Study

The assay was carried out in the absence and presence of Gen with varying concentrations of xanthine using the same method as XO activity assay [23]. The inhibition type was analyzed by using the nonlinear regression Michaelise–Menten enzyme kinetics and the corresponding Lineweavere–Burk plots. Each concentration of the inhibitor (0, 1.0, 5.0 and 10.0 μM) was with four different concentrations of substrate (25, 50, 100 and 200 μM). Each experiment was performed in triplicate.