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Inhibitory effects of daidzein and genistein on trypsin: Insights from spectroscopic and molecular docking studies

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ABSTRACT

In this work, the inhibitory effect of two isoflavonoids including daidzein and genistein on trypsin and their binding mechanism were determined by spectroscopic and molecular docking approaches. The results indicated that both daidzein and genistein reversibly inhibited trypsin in a competitive manner with IC_{50} values of 68.01×10^{-6} mol L^{-1} and 64.70×10^{-6} mol L^{-1} and K_i values of 62.12×10^{-6} mol L^{-1} and 59.83×10^{-6} mol L^{-1}, respectively. They could spontaneously bind with trypsin mainly through hydrophobic force and electrostatic interactions with a single binding site. Analysis of circular dichrosim spectra and molecular docking revealed that both isoflavonoids bound directly into the catalytic cavity and the microenvironment and secondary structure of trypsin were changed in this process, which caused the inhibition of trypsin activity. All these experimental results and theoretical data in this work would be help in understanding the mechanism of inhibitory effects of daidzein and genistein against trypsin and the potential of isoflavonoid to relieve symptoms of pancreatitis.

1. Introduction

Severe acute pancreatitis (SAP) is an inflammatory process of the pancreas that causes acinar death, local complications of even multiple organ dysfunction syndromes (MODS). Despite the availability of advanced treatment modalities for this disease, the hospital mortality rate associated with SAP is still very high (ranging from 15 to 40%) [1]. The precise pathogenic mechanism of SAP remains unclear, but the generally accepted theories include self digestion of pancreas [2], microcirculation dysfunction theory [3,4], inflammatory factors and cell mediator theory [5], bacterial translocation [6] and oxidative stress theory [7]. Among these mechanisms, the theory of self digestion of pancreas is the basic viewpoints on the cause of SAP at present. In this view, SAP occurs when pancreatic enzyme are activated prematurely (before they reach the intestine), causing auto-digestion of the pancreas. Once activated, trypsin starts a domino effect on other pancreatic enzymes. Pancreatic tissue damage activates the inflammatory response, leading to extravascular movement of serum albumin, third-spacing and pancreatic edema [8]. Based on this viewpoint, aprotinin has been used for the treatment of SAP in clinic [9]. Similarly, this view seemed to indicate that the potent trypsin inhibitory substances might have ability to relieve the symptoms of pancreatitis and could become leading compounds in the development of new drugs for pancreatitis. Recently several scientists have devoted to searching and detecting the inhibitory effects of some natural products against trypsin [10–14].

Isoflavones is a type of diphenolic secondary metabolites in plants and is generated largely in these herbs, such as *Glycine max* (soybean), *Phaseolus vulgaris* (bean), *Medicage sativa* (alfalfa), *Trifolium repens* (clovers) and *Ononis* (restharrows) [15]. Due to their beneficial effects on human health, isoflavones have been widely investigated. They are associated with reduced incidences of cardiovascular disease, some types of cancers, hyperglycaemia, Alzheimer's, menopause symptoms and diabetes mellitus types 1 and 2 [16–20]. Daidzein and genistein (structures shown in Fig. 1) are two of major bioactive isoflavone constituents of soybean, which contains Kunitz trypsin inhibitor (KTI) and Bowman-Birk inhibitor. Recently, some scientists have found that intra-gastric administration of genistein could improve the survival rate of rats with acute pancreatitis and inhibit the serum pancreatic amylase activity [21]. This result indicated that genistein could relieve the symptoms of pancreatitis by inhibiting the activity of enzyme. On the other hand, the structure of daidzein is similar to that of genistein and both of them usually present in the same herb. Therefore, it is very important to investigate the inhibitory effects of daidzein

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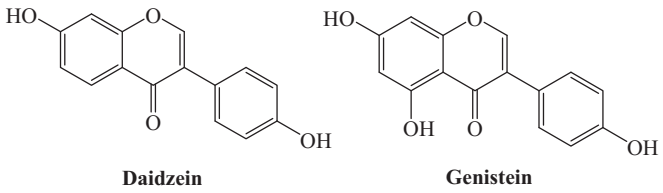

Fig 1. Molecular structure of daidzein and genistein.

and genistein on trypsin and their inhibitory mechanism. However, to the best of our knowledge, little attention was paid on the binding of daidzein and genistein to trypsin and their effects on the activity of trypsin.

In this study, the inhibitory effects of daidzein and genistein on trypsin and their interactions were investigated by spectroscopic and molecular docking approaches. The half inhibitory concentration (IC_{50}), inhibition type and inhibition constant (K_i) were determined. The binding constant, thermodynamic parameters, binding site and the effect of daidzein and genistein on trypsin conformation were evaluated. The aim of this study was to determine the inhibitory mechanism of daidzein and genistein on trypsin activity, and to provide useful information for clinical applications of daidzein and genistein as trypsin inhibitors.

2. Materials and methods

2.1. Materials

Trypsin, *N*α-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BApNA) and casein were purchased from Sigma-Aldrich Chemical Co. (USA) and used without further purification. Trypsin solution (1.0×10^{-3} mol L^{-1}) was prepared in sodium phosphate buffer (0.02 mol L^{-1}, pH = 7.4) and then diluted to the required concentrations with the buffer. Daidzein and genistein were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and was prepared by dissolving their crystals in methanol to form 1.0×10^{-3} mol L^{-1}, and then was kept in the dark at 4 °C. All other chemicals were of analytical grade and used without further purification.

2.2. Trypsin inhibition assay and enzyme kinetics

The enzyme activity was examined at room temperature by monitoring the absorbance at 410 nm accompanying the hydrolysis of substrate BApNA. Briefly, 100 μL of trypsin (1.0×10^{-5} mol L^{-1}) was incubated with various concentrations of isoflavonoid for 10 min at room temperature. Then, 200 μL of BApNA (20 mmol L^{-1}) and 300 μL of 0.02 mol L^{-1} sodium phosphate buffer (containing 20 mmol L^{-1} $CaCl_2$, pH = 7.4) were added and adjusted to 1.0 mL with distilled water. The enzymatic activity was measured by continuously monitoring the absorbance of ρ-nitroaniline at 410 nm every 20 s for 5 min. The enzymatic relative activity assayed without isoflavonoid was defined as 100%. Relative enzymatic activity(%) = (slope of reaction kinetics equation obtained by reaction with inhibitor)/(slope of reaction kinetics equation obtained by reaction without inhibitor) × 100.

According to the experiment above, the constant of inhibition (K_i) for trypsin was determined by the method of Wang with some modification [22]. A volume of 100 μL of trypsin (1.0×10^{-3} mol L^{-1}) was incubated with isoflavonoid at different daidzein/genistein final concentrations (0, 2.0×10^{-5}, 4.0×10^{-5} and 8.0×10^{-5} mol L^{-1}) in the reaction system at room temperature for 10 min. The enzymatic activities were measured at the different concentrations of substrate BApNA (5.0×10^{-4}, 6.7×10^{-4}, 1.0×10^{-3} and 2.0×10^{-3} mol L^{-1}). The inhibitory type can be described by the Lineweaver-Burk equation in double reciprocal form [23]:

$$\frac{1}{v} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \quad (1)$$

$$K_m^{app} = K_m \left(1 + \frac{[I]}{K_i}\right) \quad (2)$$

where v is the enzyme reaction rate in the absence and presence of isoflavonoid. K_m and K_m^{app} are the Michaelis-Menten constant and the apparent Michaelis-Menten constant, respectively. K_i is the inhibition constant. [I] and [S] are the concentrations of inhibitor and substrate, respectively.

2.3. Fluorescence spectroscopy

All fluorescence spectra of pepsin were recorded on Hitachi F-4500 spectrofluorimeter (Hitachi, Japan) equipped with 1.0 cm quartz cells. The experimental temperature was maintained by recycling water throughout the quartz cell. Fluorescence emission spectra were recorded over a wavelength range of 300–450 nm at an excitation wavelength of 280 nm, the scan speed was set at 500 nm min^{-1} and the slit widths of emission and excitation were set at 5.0 nm and 5.0 nm, respectively.

It is important to keep in mind that phenolic compounds absorb energy at both protein excitation and emission wavelength. Consequently, an inner filtering effect occurs, which intensifies with increasing concentration of phenolic compounds. To overcome this effect, the fluorescence intensity was corrected according to Beer-Lambert's law using the following equation [24]:

$$F_{cor} = F_{obs} \times 10^{(A_{ex}+A_{em})/2} \quad (3)$$

where F_{cor} and F_{obs} are the fluorescence intensity corrected and observed, respectively. A_{ex} and A_{em} are the sum of the absorbance of isoflavonoid in fluorescence determination system at excitation wavelength and the emission wavelength, respectively.

2.4. Circular dichroism spectroscopy

The Circular dichroism (CD) spectra of trypsin and its complexes were recorded with a JASCO (J-810) spectropolarimeter (Tokyo, Japan) with a 1.0 mm quartz cuvette in the range of 190–260 nm. Two sets of isoflavonoid-trypsin complexes were prepared at different molar ratios of isoflavonoid to trypsin of 0:1 and 4:1. The data were collected with an interval of 1.0 nm and a time-per-point of 0.5 s at room temperature. The corresponding buffer solution was used for baseline correction.

2.5. Molecular docking study

Molecular simulation based on docking was performed using Autodock 4.0 software package. For the docking studies the structures of daidzein and genistein were generated from Chemdraw software. The known crystal structure of trypsin (PDB ID: 2ZQ1) was obtained from the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). Autodock 4.0 suite of programs which utilizes the Lamarckian Genetic Algorithm was implemented for the docking studies of pepsin and insecticides. In the initial stage of docking, all the water molecules were removed and the hydrogen atoms were added followed by computing Gasterger charges, as required in Lamarckian Genetic Algorithm. For the docking analysis the grid size was set to 70 Å, 70 Å and 70 Å along X, Y and Z-axis with 0.375 Å grid spacing. The docking parameters used were as follows: GA population size = 100 and maximum number of energy evaluation = 2,500,000, other parameters used were default values. The