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Investigation on the binding interaction between silybin and pepsin by spectral and molecular docking

Hua-jin Zeng [a], Jing You [a], Hui-li Liang [a], Tingting Qi [a], Ran Yang [b,*], Ling-bo Qu [b,c]

[a] School of Pharmaceutical Sciences, Zhengzhou University, Zhengzhou 450001, PR China
[b] The College of Chemistry and Molecular Engineering, Zhengzhou University, Zhengzhou 450001, PR China
[c] School of Chemistry and Chemical Engineering, Henan University of Technology, Zhengzhou 450001, PR China

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ABSTRACT

In this study, the binding mode of silybin with pepsin was investigated by spectroscopic and molecular docking methods. Silybin can interact with pepsin to form a silybin–pepsin complex. The binding constant, number of binding sites and thermodynamic parameters were measured, which indicated that silybin could spontaneously bind with pepsin mainly through hydrophobic interaction with one binding site. Molecular docking results revealed that silybin bound into the pepsin cavity site. Synchronous fluorescence and three-dimensional fluorescence results provide data concerning conformational and some micro-environmental changes of pepsin. Furthermore, in order to reveal whether the binding process can inhibit the activity of pepsin in vivo, the effect of silybin on pepsin activity in rat was investigated. The present study provides direct evidence at a molecular level to show that exposure to silybin could induce changes in the enzyme pepsin structure and function.

1. Introduction

Pepsin, the main digestive enzyme in gastric juice, is responsible for the most of digestive activities in the stomach [1]. It is very probable for drug to interact with pepsin when it enters the stomach. Meanwhile, the activity of pepsin may also be affected by the drug. Therefore, study on the interaction of drug and pepsin is helpful for us to understand the drug's absorption in the stomach, which is related to the drug's biological performance. Recently, the interactions between some compounds with pepsin have been reported [2–4].

Silybin (structure shown in Fig. 1) is the major biologically active component of an extract from the seeds of the milk thistle (*Silybum marianum* (L.) Gaertn) known as silymarin [5]. Silybin possesses hepatoprotective ability and has been widely used as a natural remedy in therapy of various acute and chronic liver diseases as well as in protection of the liver from numerous hepatotoxins and mycotoxins [6–8]. However, due to the slight solubility of silybin in water, the permeation across the intestinal epithelial cells and the gastrointestinal tract absorption were poor [9,10]. Therefore, many efforts were made to increase of oral bioavailability and improve the biological effects of silybin [11,12]. On the other hand, based on the data on file with the manufacture of silybin, some adverse effects, including vomiting, hiccup singultation and so on, would occur in some patients even if they took the normal dosage (about 3.5–7.0 mg/kg). The reason for this might be due to indigestion caused by silybin. Therefore, as a promising natural drug for the cure of hepatitis, it is very necessary for us to learn about the knowledge that whether silybin could interaction with pepsin, what was the main interaction force between them and would such an interaction affect the activity of pepsin.

In this study, the interaction behavior between silybin and pepsin has been investigated by fluorescence spectroscopy, synchronous fluorescence spectroscopy, three-dimensional fluorescence spectra and molecular modeling. Furthermore, to reveal whether the binding process can inhibit the activity of pepsin in vivo, the effect of silybin on pepsin activity was also investigated in rats.

2. Experimental

2.1. Reagents

The porcine pepsin and bovine hemoglobin were purchased from Sigma–Aldrich Chemical Co. (USA) and silybin and L-tyrosine were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All the chemicals were of analytical-reagent grade and used without

* Corresponding author. Tel.: +86 371 67756886.
E-mail address: qulingbo@zzu.edu.cn (R. Yang).

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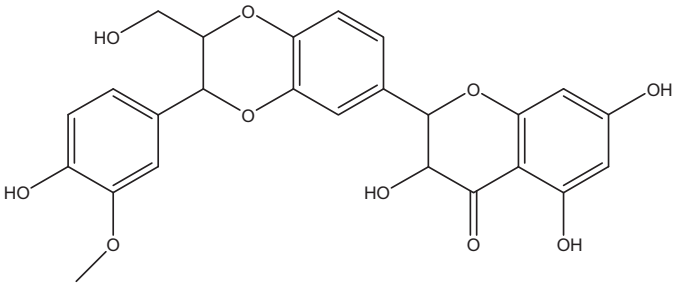

Fig. 1. Molecular structure of silybin.

further purification. Silybin was dissolved in methanol to form a 1.0×10^{-3} mol L^{-1} solution, which was used to determine the binding sites of silybin on pepsin. Citric acid-sodium citrate buffer solutions (0.2 mol L^{-1}) containing 0.1 mol L^{-1} NaCI were prepared to adjust the acidity of the system pH 2.0, which is the most common pH for pepsin digests. Water was purified with a Milli-Q purification system (Barnstead, USA).

2.2. Equipment and spectral measurements

The fluorescence spectra were recorded on Hitachi F-2500 spectrofluorimeter equipped with 1.0 cm quartz cells. The excitation wavelength was 280 nm for all cases with an excitation and emission band pass (slit) of 10 nm. The experimental temperature was maintained by recycling water throughout the quartz cell. The UV–vis spectrum was recorded at room temperature on a Shimadzu UV-2450 spectrophotometer (Shimadzu, Japan) equipped with 1.0 cm quartz cells.

2.3. Procedures

The fluorescence measurements were carried out by successive addition of the solution of silybin to a fixed amount of pepsin (to give a final concentration of 2.5×10^{-5} mol L^{-1}) in each tube. The final volume was made up to 5.0 mL with citric acid-sodium citrate buffer (pH 2.0). Thus, a series of solutions containing different amount of silybin and a definite amount of pepsin were obtained. The fluorescence spectra were then measured (excitation at 280 nm and emission wavelengths of 290–450 nm) at 293 and 310 K, respectively. All solutions were mixed thoroughly and kept for 20 min before measurement.

The synchronous fluorescence spectra of pepsin in the presence of silybin were recorded at 293 K and the *D*-value ($\triangle\lambda$) between excitation wavelength and emission wavelength were stabilized at 15 or 60 nm. The three dimensional fluorescence spectra were performed under the following conditions: the emission wavelength range was selected from 270 to 500 nm, the initial excitation wavelength was set to 200 nm, and the scanning number was 15 with the increment of 10 nm. The UV–vis absorbance spectra of silybin–pepsin system were recorded at 293 K.

2.4. Molecular docking investigation

Docking calculations were carried out using AutoDock 4.0. The structure of silybin was generated by Chem 3D Ultra and optimized by density functional theory at B3lyp/6-31 + g (d) level implemented in Gaussian 03 until all egienvalue of the Hesssian matrix were positive. With the aid of AutoDock 4.0, the ligand root of silybin was detected and rotatable bonds were defined.

The crystal structure of pepsin (protein ID: 5PEP) was downloaded from Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). All water molecules were removed and the polar hydrogen and the Gasteiger charges were added at the beginning of docking study. To recognize the binding sites in pepsin, docking was carried out with setting of grid box size 90 Å × 100 Å × 90 Å along *x*, *y*, *z* axes covering whole protein with Kollman charges. The grid center was set at −17.802, 40.376 and 86.848 Å. At first, AutoGrid was run to generate the grid map of various atoms of the ligand and receptor. After the completion of grid map, ligand flexible docking simulations were performed with 100 runs and 2,500,000 energy evaluations, 27,000 numbers of generations, 50 GA populations and root mean square cluster tolerance 2.0 Å per run. Finally, the lowest energy conformation was used for docking analysis.

2.5. Effect of silybin on the activity of pepsin in rats

2.5.1. Preparation of calibration curve of L-tyrosine

The standard stock solution of L-tyrosine was prepared by dissolving appropriated quantities of L-tyrosine in 0.04 mol L^{-1} HCI solution and storing at 4 °C in a refrigerator. Before use, the stock solution was diluted with 0.04 mol L^{-1} HCI solution to prepare standard solutions with a series of concentrations (20, 40, 60, 80 and 100 μg mL^{-1}) for the construction of the calibration curve. 1.0 mL of standard soltuion was added to 5.0 mL 0.5 mol L^{-1} Na_2CO_3 and 0.5 mL Fehling's solution and mixed. After putting statically in a room temperature for 20 min, the value of OD_{660} of the mixed solution was measured by a spectrophotometer. The calibration curve of L-tyrosine was constructed by plotting the value of OD_{660} with concentration of L-tyrosine ($A = 0.0094C + 0.00503$, $R^2 = 0.9990$).

2.5.2. Animals and drug administration

20 male and female Sprague–Dawley rats (200 ± 5 g) were obtained from Henana Laboratory Animal Centra (Zhengzhou, China) and were divided into 4 groups evenly. They were kept in an environmentally controlled breeding room for 5 days before starting the experiments and fed with standard laboratory food and water. After 12 h of fasting, one group was orally administered water and the others were administered silybin at a dose of 5, 10 and 20 mg kg^{-1}, respectively.

2.5.3. Collection of gastric juice and pepsin activity measurement

After 4 h of oral administration, the rats were sacrificed to obtain the stomach and then the gastric juice was taken and put into centrifuging tubes. After centrifugation at 4000 rpm for 10 min, the supernatant was transferred to Eppendorf tube for activity experiment.

The enzyme activity was detected by the method detailed by Anson and Li with some modifications [13,14]. 0.5 mL of supernatant was diluted 30-folds with 0.04 mol L^{-1} HCI solution. And then, 0.5 mL of the mixture was pipetted accurately to a tube and 2.0 mL of 5% bovine hemoglobin solution was added in. After incubation at 37 °C for 10 min, 5.0 mL of 5% trichloroacetic acid was added to terminate the reaction. The mixture was put there for 30 min statically, and then centrifuged at 4000 rpm for 10 min. After addition of 5.0 mL of 0.5 mol L^{-1} Na_2CO_3 and 0.5 mL of Fehling's solution to the supernatant, the mixture was incubated at room temperature for 20 min and then the value of OD_{660} was measured using a spectrophotometer. The concentrations of L-tyrosine (C) in each sample can be calculated from calibration curve of L-tyrosine. And the activity of pepsin can be calculated by the following equation:

Pepsin activity (μg mL^{-1} min^{-1}) = $(C_{sample} - C_{control}) \times 30 \times 25/10$ min.