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Spectroscopic and molecular modeling investigation on the interactions between hyaluronidase and baicalein and chrysin

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ABSTRACT

In the work described on this paper, the bindings of baicalein and chrysin with hyaluronidase (HAase) were studied by fluorescence, synchronous fluorescence, three-dimensional fluorescence and molecular docking methods. The results indicated that both of flavones could interact with HAase to form flavone–HAase complexes. The binding constant, number of binding sites and thermodynamic parameters were measured at different temperature, which indicated that flavones could spontaneously bind with HAase through electrostatic forces with one binding site. Based on synchronous and three-dimensional fluorescence spectra and the molecular docking results, both of flavones bound directly into the enzyme cavity site and the binding of flavones into the enzyme cavity influenced the microenvironment of the HAase activity site which resulted in the reduced HAase activity. The present study provides direct evidence at a molecular level to understand the mechanism of inhibitory effect of flavone against HAase and explain the anti-inflammatory mechanism of *Scutellaria baicalensis* Georgi as an anti-inflammatory drug.

1. Introduction

Hyaluronidase (HAase), an enzyme which depolymerize or hydrolyse hyaluronic acid presumably by splitting glucosaminidic bonds to yield oligosaccharides, was found both in organs and in body fluids [1]. This enzyme was earlier claimed to be involved in allergic effects, migration of cancer cells, inflammation, petechial hemorrhages following its injection in mesentery preparations and also the increase in permeability of the vascular system [2]. This information seemed to indicate that HAase might be present as one of the target enzyme of influent calcium ion in the mast cell and might directly control the mast cell degranulation [3]. In addition, this information also seemed to suggest that potent HAase inhibitory substances might have anti-allergic, anti-inflammatory and anticancer effects, and could become leading compounds in the development of new anti-allergic and anti-inflammatory drugs [4]. Therefore, on the basis of this information, some researches began to search and detect the inhibitory effect of some natural products against HAase [5–7].

Scutellaria baicalensis Georgi (namely Huang-Qin in Chinese), a traditional Chinese medicine, has been widely used to treat various diseases including inflammation, hypertension, cardiovascular disease, and bacterial and viral infections [8,9] and has officially been listed in the China Pharmacopeia as a medicinal plant [10]. Baicallein (5,6,7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one, Fig. 1) is a major bioactive flavone constituent of *S. baicalensis* and possesses a wide range of biological activities, including anti-cancer [11,12], antioxidant [13], anti-allergic and anti-inflammatory effects [14]. Chrysin (5,7-dihydroxyflavone, Fig. 1), which is an another flavone found abundantly in *S. baicalensis*, has received attention because of its potent antioxidant and anti-inflammatory properties [15]. This information indicated that baicalein and chrysin might possess potential ability to inhibit the activity of HAase. In previous studies, several flavonoids, including kaempferol, quercetin and rutin, had been proved to possess the inhibitory effect on HAase [16,17]. However, to our knowledge, these studies were limited to the enzymatic activity assay and the inhibitory effects of baicalein and chrysin on HAase and the inhibitory mechanism of them has not been investigated.

In the present work, the inhibitory effect and interaction mechanism of baicalein and chrysin with HAase were investigated by spectroscopic and molecular modeling methods. The binding constant, thermodynamic parameters, the special binding site and

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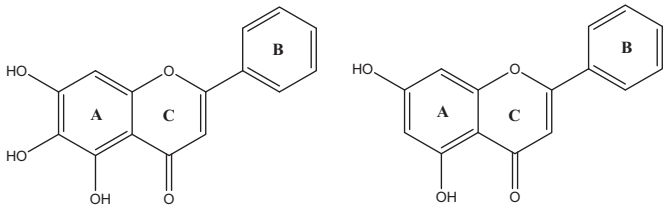

Fig. 1. Molecular structure of baicalein and chrysin.

the effect of baicalein and chrysin on HAase conformation were evaluated. The aim of this work was to provide direct evidence at a molecular level to understand the mechanism of inhibitory effect of these two flavones against HAase and explain the anti-inflammatory mechanism of *S. baicalensis* Georgi.

2. Materials and methods

2.1. Materials

Bovine testicular HAase (EC3.2.1.35) was purchased from Sigma–Aldrich Chemical Co. (USA) and its stock solution (1.0×10^{-5} mol L^{-1}) was made in sodium phosphate buffer (0.2 mol L^{-1}, pH = 7.4) and then diluted to the required concentrations with the buffer. Baicalein and chrysin were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and its stock solution (1.0×10^{-3} mol L^{-1}) was prepared by dissolving its crystals in methanol and constant-volume with the sodium phosphate buffer. 10% *p*-Dimethylaminobenzaldehyde (DMAB) was prepared by dissolving 1.0 g DMAB in 12.5% HCl solution and then diluted with acetic acid to 100 mL. All reagents and solvents were of analytical reagent grade, and ultra-pure water was used throughout the experiment. All stock solutions were stored at 4 °C.

2.2. Apparatus

The UV–vis absorption measurements were performed with a Shimadzu UV-2450 spectrophotometer equipped with 1.0 cm quartz cells. Fluorescence spectra were recorded on a Hitachi spectrofluorimeter Model F-2500 equipped with 1.0 cm quartz cells. All experiments, unless otherwise specified, were carried out at room temperature.

2.3. Methods

2.3.1. HAase activity assay

Effect on HAase enzyme activity was measured by the modified Morgan–Elson method with some modifications [3,16]. Briefly, 0.5 mL HAase enzyme solution (500 U/mL) was incubated with the test samples for 20 min at 37°C. Then, 1.0 mL calcium chloride (12.5 mmol L^{-1}) was added to the mixture and incubated for 20 min at 37°C again. 0.5 mL hyaluronic acid sodium solution (0.5 mg/mL) was added and incubation at 37°C for 40 min. After the addition of 0.1 mL sodium hydroxide (0.5 mol L^{-1}) and 0.5 mL sodium borate (0.1 mol L^{-1}), the reaction mixture was heated in a boiling water bath for 5 min to stop the enzyme reaction. After cooling to room temperature, 3 mL of 10% DMAB solution was added and incubated at 37°C for 30 min when color developed. The absorbance at 530 nm of the clear supernatant was measured. Test samples were replaced by the buffer solution for the control. The HAase inhibition rate was calculated using the following formula:

$$\text{Inhibition rate}\,(\%) = (\mathrm{OD}_{530\,\text{control}} - \mathrm{OD}_{530\,\text{sample}})/\mathrm{OD}_{530\,\text{control}} \times 100.$$

2.3.2. Fluorescence measurement

The fluorescence measurements were carried out by successive addition of the solution of flavone (baicalein or chrysin) to 1.0 mL of 1.0×10^{-5} mol L^{-1} HAase solution in each tube. The final volume was made up to 5.0 mL with sodium phosphate buffer. Thus, a series of solutions containing different amount of flavone and a definite amount of HAase (a final concentration of 2.0×10^{-6} mol L^{-1}) 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 HAase in the presence of flavone were recorded at 293 K and the *D*-value ($\Delta\lambda$) 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.

2.3.3. Molecular docking investigation

The docking program AutoDock 4.0 was used to explore the probable interaction between flavone (baicalein or chrysin) and HAase. The 3D structures of baicalein and chrysin were generated in Chem3D Ultra 8.0, and the crystal structure of HAase (PDB ID: 2PE4) was retrieved from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). To carry out docking simulations, a grid box was defined to enclose the active site with dimensions of 126 Å × 126 Å × 126 Å and a grid spacing of 0.375 Å. The grid maps for energy scoring were calculated using AutoGrid. Docking calculations were performed using the Lamarckian genetic algorithm (LGA) and the search parameters were set to 100 times.

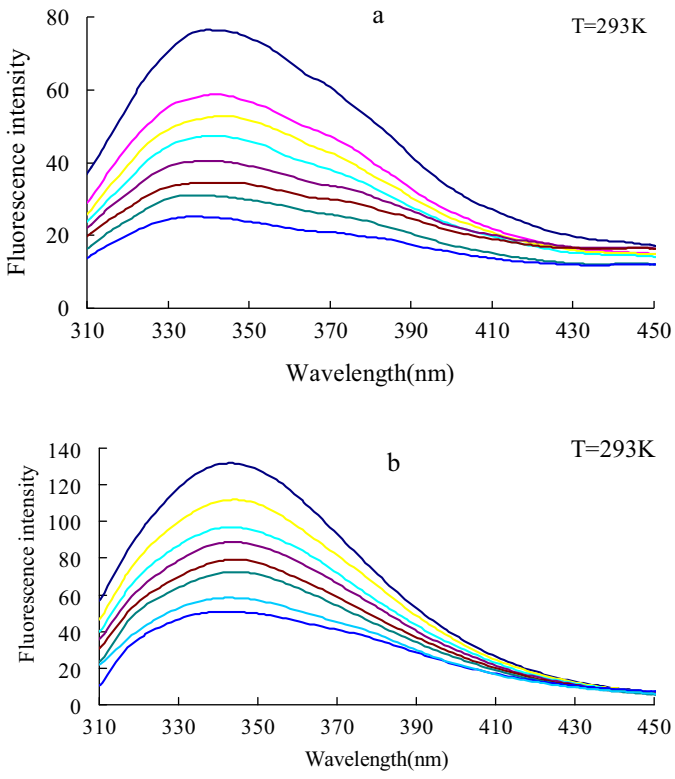

Fig. 2. The fluorescence emission spectra of HAase in the presence of increasing amounts of baicalein (a) and chrysin (b). Peak from up to down $C_{\text{baicalein}}$ = (0, 4.0, 8.0, 12.0, 16.0, 20.0, 24.0, 30.0) × 10^{-6} mol L^{-1}, C_{HAase} = 1.0×10^{-6} mol L^{-1}; C_{chrysin} = (0, 6.0, 12.0, 16.0, 18.0, 24.0, 30.0, 36.0, 42.0) × 10^{-6} mol L^{-1}, C_{HAase} = 2.0×10^{-6} mol L^{-1}.