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Inactivation of jack bean urease by scutellarin: Elucidation of inhibitory efficacy, kinetics and mechanism

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ABSTRACT

In the present study, the inactivation effect of scutellarin (SL) on jack bean urease was investigated to elucidate the inhibitory potency, kinetics and mechanism of inhibition. It was revealed that SL acted as a concentration- and time-dependent inactivator of urease characteristic of slow-binding inhibition with an IC_{50} of 1.35 ± 0.15 mM. The rapid formation of the initial SL–urease complex with an inhibition constant of $K_i = 5.37 \times 10^{-2}$ mM was followed by a slow isomerization into the final complex with the overall inhibition constant of $K_i^* = 3.49 \times 10^{-3}$ mM. High effectiveness of thiol protectors, such as L-cysteine (L-cys), 2-mercaptoethanol (2-ME) and dithiothreitol (DTT) significantly slowed down the rate of inactivation, indicating the strategic role of the active site sulfhydryl group in the blocking process. While the insignificant protection by boric acid and fluoride from the inactivation further confirmed that the active site cysteine should be obligatory for urease inhibition, which was also rationalized by the molecular docking study. The inhibition of SL on urease proved to be reversible since SL-blocked urease could be reactivated by DTT application and multidilution. The results obtained indicated that urease inactivation resulted from the reaction between SL and the sulfhydryl group.

1. Introduction

Urease (urea amidohydrolase, EC 3.5.1.5), widespread in plants, fungi and bacteria [1], is a nickel-dependent metalloenzyme that catalyzes the hydrolysis of urea to ammonia and carbon dioxide [2]. Though of different origin, all ureases share highly conserved tertiary structures, common amino acid sequences of the active site and consequently similar catalytic mechanisms [3–5]. Among various ureases, jack bean (*Canavalia ensiformis*) urease, the first enzyme crystallized [6] and best-characterized [7–9], was widely empolyed in urease inhibition research [10,11]. Jack bean urease is a homohexamer thiol-rich enzyme with 15 cysteine residues per subunit. Pivotal catalytic characteristic common to ureases are the nickel ions (Ni^{2+}) and the sulfhydryl groups, especially the multiple cysteinyl residues bearing thiol groups in the active site of the enzyme. These provide opportunities for different possible ways for the urease inhibitor interaction. Numerous studies have pointed out that urease sulfhydryl groups were responsible for urease inactivation [12,13].

Urease, releasing an abnormally large amount of ammonia, led to a chain of deleterious complications, especially in agriculture and medicine. In agriculture, high urease activity causes excessive amounts of ammonia emission into the atmosphere during urea fertilization which bring about significant environmental and economic problems [14–16]. In medicine, some bacterial ureases served as an important virulence factor implicated in the development of kidney stones, pyelonephritis, peptic ulcers, and other clinical complications [4,17].

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A variety of urease inhibitors have been investigated in the past decades, including phosphoramidates [2], hydroxamic acids [2,18], boric and boronic acids [19], heavy metal ions [20], quinones [21] and imidazoles [22]. However, most of these compounds are too toxic or unstable to be therapeutic agents. Thus, current researches are focused on finding novel urease inhibitors with promising levels of activity from natural plant.

Polyphenols, such as flavones and isoflavones, constitute one of the vital classes of compounds in higher plants. To date, vast evidences established that polyphenols exhibited broad variety of biological activities, including inhibitory properties against ureases, such as quercetin [11], rutin [23] and lawsonaringenin [24]. Scutellarin (SL, $C_{21}H_{18}O_{12}$, showed in Fig. 1), a flavone glucuronide of 5,6,4′-trihydroxyflavone-7-O-glucoronide, is the primary effective constituent of traditional Chinese medicinal herb *Erigeron breviscapus* (Vant.) Hand-Mazz. SL was also a major active component of breviscapine, which has been widely used to treat cardiovascular and cerebrovascular diseases in clinical practice [25]. Pharmacological studies have demonstrated that it possessed remarkable biological activities including anti-oxidative [26], anti-inflammatory [27], vasodilatory [25], cardiovascular and cerebrovascular ischemia protective effects [28,29], as well as anti-HIV-1 efficacy[30].

In the present work, urease inhibitory potency by SL was demonstrated for the first time. In an effort to explore the possible mechanism underlying the urease inhibitory action of SL, the kinetics and specific inhibitory mechanism were investigated. Firstly, we carried out residual activity assay and inhibition progress curves analysis to evaluate the inhibitory effect and specific kinetics of SL on jack bean urease. Secondly, we conducted the protective experiments by thiols and competitive inhibitiors, SL-thiol-urease interacting analysis and reactivation test to identify the targeted active site (Ni^{2+} and/or the sulfhydryl group). Thirdly, to give further explanation for the deductive inhibitory mechanism, we performed molecular docking program to explore the possible binding mode of SL and jack bean urease.

2. Materials and methods

2.1. Materials

Scutellarin ($C_{21}H_{18}O_{12}$, CAS number: 27740-01-8) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

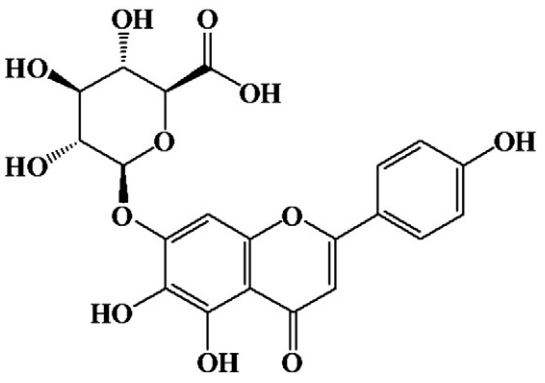

Fig. 1. Chemical structure of scutellarin.

Urease (from jack beans, type III, specific activity 40.3 units/mg solid, the total content of reducing agents less than 1.5 μg/U and used without further purification), urea (Molecular Biology Reagent), D,L-dithiothreitol (DTT), 2-mercaptoethanol (2-ME), L-cysteine (L-cys), boric acid and sodium fluoride (NaF) were purchased from Sigma Aldrich. Other chemicals were obtained from Guangzhou Chemical Reagent Factory (China). All reagents used were of analytical grade. Phosphate buffer 20 mM, pH 7.0 was prepared by adjusting pH of phosphoric acid with NaOH. 2 mM EDTA was added to all enzyme-containing solutions.

2.2. Standard urease activity assay

The standard urease assay mixture (25 mL) consisted of 50 mM urea in 20 mM phosphate buffer, pH 7.0 and 2 mM EDTA. The reactions were initiated by addition of small aliquots of enzyme-containing (0.25 mg) solution. The assay was run for 20 min and the enzyme activity was determined by measuring the concentration of the ammonia released. For that, samples were withdrawn from the reaction mixtures and the ammonia was determined at 595 nm by the spectrophotometric modified Berthelot (phenol-hypochlorite) method [31] at ambient temperature. The activity measured for uninactivated urease was accounted as a control activity of 100%. All experiments were triply repeated.

2.3. Determination of K_M and v_{max}

The Michaelis constant K_M and the maximum velocity v_{max} in the absence of the inhibitor were determined by measuring the initial reaction velocities at different urea concentrations ranging from 2 mM to 50 mM. The values obtained by applying nonlinear regression to the Michaelis–Menten equation were: $K_M = 7.10 \pm 0.10$ mM and $v_{max} = 1.15 \pm 0.04$ mM/min.

2.4. Residual activity measurement

A concentrated solution of urease was preincubated with serial concentrated solution of SL (0.95–2.25 mM) at 37 °C in the absence of urea. The preincubation mixture comprised 0.25 mg/mL urease, 20 mM phosphate buffer, pH 7.0, 2 mM EDTA. The time when the enzyme and the inhibitor were mixed was marked as zero time of incubation. After appropriate periods of time, aliquots from the preincubation mixture were withdrawn and transferred into the standard assay mixtures for urease residual activity determination. The activity measured for uninactivated urease was accounted as a control activity of 100%.

2.5. Kinetic analysis of urease inhibition by SL

The progress of urease inactivated by SL was investigated in 20 mM phosphate buffer, pH 7.0, 2 mM EDTA. The initial concentration of urea in the reaction mixture was 50 mM and the concentration of urease was 0.25 mg/mL in all assay. The reaction was activated in the absence and presence of SL by virtue of two procedures:

(1) The progress curves were determined for the reactions initiated by the addition of the enzyme into the reaction mixtures containing different concentrations of SL (0.95, 1.27, 1.69, 2.25 mM).