Kinetics Studies on Symplocomoside: A Urease Inhibitor

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Summary: The mechanism of inhibition of jack bean and *Bacillus pasteurii* ureases was investigated by symplocomoside (**1**) which is a phenolic glycoside of salirepin series and has been isolated from *Symplocos racemosa* Roxb. Lineweaver-Burk, Dixon plots and their secondary replots showed that **1** was a non-competitive inhibitor of these enzymes. *Ki* values were found to be 77.60 ± 1.22 μM and 63.47 ± 0.92 μM against jack bean and *Bacillus pasteurii* ureases respectively.

Introduction

Symplocos racemosa Roxb (Symplocaceae) is known as "Lodhra" and is used in the Indian system of medicine as single drug or in multicomponent preparations (viz., "Lodhrasava"). Medicinally the bark is used as an acrid, digestible, astringent for the bowels and is useful in eye diseases, spongy gums and for bleeding. The bark also shows depressant action on blood pressure and intestinal movements and is widely used as an Ayurvedic remedy mainly for gynecological disorders and for ulcers of vagina. Unani medicine uses it as emmenagogue and aphrodisiac. It is a potent remedy for inflammation and cleaning uterus and is also used to treat leucorrhea and menorrhagia [1]. The study on the bark of *Symplocos racemosa* Roxb. revealed that its aqueous extract on oral administration significantly stimulated serum follicle-stimulating hormone (FSH) level ($P < 0.016$) along with the rise in serum luteinizing hormone (LH) level ($P < 0.001$). Moreover, histopathological studies revealed enhanced folliculogenisis, presence of mature follicles and detached oocytes, which were the result of increased FSH and LH levels. All these results were in concordance with the traditional use of this plant for female disorders [1].

Urease (urea amidohydrolase, EC: 3.5.1.5) occurs throughout the animal and plant kingdom. Many microorganisms use this enzyme to provide a source of nitrogen for growth and the enzyme plays an important role in plant nitrogen metabolism during the germination process [2, 3]. The presence of urease activity in soils is exploited in the widespread agricultural practice of urea-based fertilizer application for enhancing crop yields. Unfortunately, excessive levels of soil urease can degrade fertilizer urea too rapidly and result in phytopathic effects and loss of volatilized ammonia [4]. Of medical and veterinary interest, urease is a virulence factor in certain human and animal pathogens; it participates in the development of kidney stones, pyelonephritis, peptic ulcers and other disease states [5]. The obvious remedy for treating bacterial infection with antimicrobials, however, has often proven futile [6] and only a few combination regiments have reached clinical practice. Thus the need for alternative or novel treatment is evident. Consequently, the discovery of potent and safe urease inhibitors has been a very important area of pharmaceutical research due to the involvement of ureases in different pathological conditions. We have previously reported a number of novel synthetic and natural inhibitors of urease, their inhibition kinetics and structure-activity relationship studies [7-10]. In continuation of this work to discover new and potent inhibitors of medicinally important enzymes through high-throughput screening assays, we identified symplocomoside (**1**) as an effective inhibitor of jack bean and *Bacillus pasteurii* ureases. The objective of the current investigation is to explore the possible binding interactions of **1** in the target protein in search for an effective inhibitor of urease.

Results and Discussion

Symplocomoside (**1**) which is a phenolic glycoside of salirepin series was isolated as a white powder from the ethyl acetate soluble fraction of *Symplocos racemosa* Roxb. and its structure (Fig. 1) was deduced through extensive spectral studies [11].

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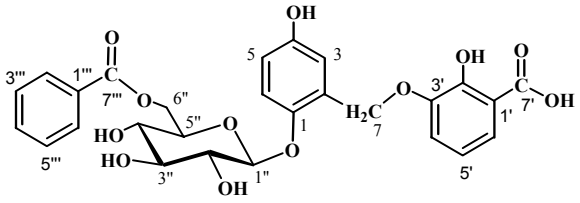


Fig. 1: Structure of symplocomoside (**1**).

Urease is an enzyme that is present in many plants and in soil that catalyzes the hydrolysis of urea to ammonium and carbamate ions, which decompose to carbon dioxide and ammonia. The active site contains two nickel (II) atoms which, as shown by X-ray analysis, are linked by a carbamate bridge; furthermore, two imidazole nitrogen atoms are bound to each nickel atom and a carboxylate group and a water molecule fill the remaining coordination site of the metal ion [5]. The coordination geometry of the first nickel atom is pseudo tetrahedral, while that of second is roughly trigonal bipyramidal. In order to discriminate among the inhibition capacities of various compounds, it is important to understand the coordination mechanism between the active site of the enzyme and the inhibitor.

Symplocomoside (**1**) inhibited urease enzymes in a concentration-dependent manner with K_i values of 77.60 ± 1.22 μM and 63.47 ± 0.92 μM against jack bean and *Bacillus pasteurii* ureases, respectively (Table-1). K_i values were calculated in three different ways; first, the slopes of each line in the Lineweaver-Burk plot were plotted against different concentrations of **1**; second, the $1/V_{maxapp}$ was calculated by plotting different fixed concentrations of urea versus ΔV in the presence of different fixed concentrations of **1** in the respective assays of urease. Then K_i was calculated by plotting different concentrations of inhibitor versus $1/V_{maxapp}$. K_i was the intercept on the x-axis. In the third method, K_i was directly measured from Dixon plot as an intercept on x-axis (Fig. 2). Determination of the inhibition type is important in understanding the mechanism of inhibition and the sites of inhibitor binding. Lineweaver-Burk, Dixon plots and their replots indicated that **1** is a non-competitive inhibitor of jack bean and *B. pasteurii* ureases, as in its presence, there was a decrease in V_{max} without affecting the affinity (K_m values) of the urease towards the substrate (urea). In other words, **1** and urea bind randomly and independently at the different sites of urease indicating that inhibition depends only on the concentration of **1** and dissociation constant (K_i). These mechanistic studies of **1** are expected to provide rational information for the design of a new potential inhibitor of jack bean and *B. pasteurii* ureases.

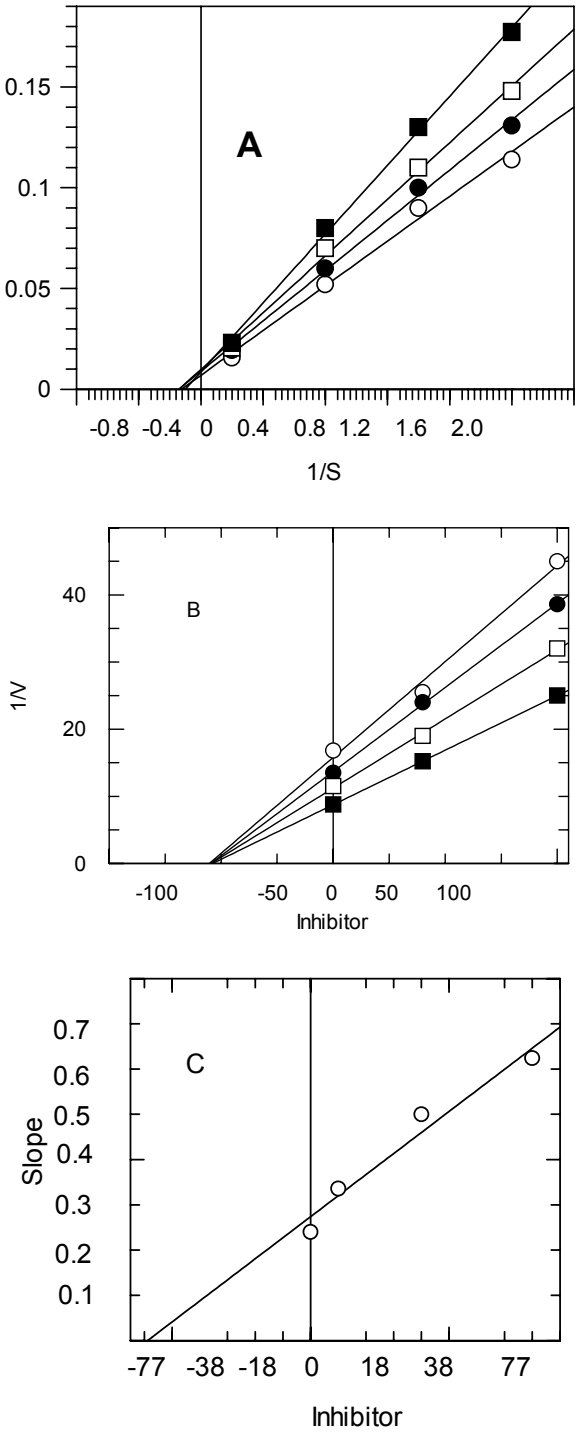


Fig. 2: Steady state inhibition of J.B and B.P ureases by symplocomoside (**1**).
A. Lineweaver– Burk plot of reciprocal of initial velocities versus reciprocal of four fixed J.B urease concentrations in absence (○) and presence of 50.0 μM (●), 75.0 μM (□), 100 μM (■) of **1**. **B** is the Dixon plot of reciprocal of the initial velocities versus

various concentrations of **1** at fixed urease concentrations, (■) 24 μM, (□) 18.0 μM, (●)12.5 μM and (○) 6.2 μM. **C.** is Dixon Secondry plot slope vs three different concentration of **1**.

Table-1: *In vitro* inhibition of ureases by symplocomo-side (**1**).
(a) Standard mean error of 3-5 assays, (b) K_i is the mean of three values calculated by using the Dixon plot and Lineweaver-Burk secondary plots, (c) J.B is jack bean urease and B.P is *Bacillus pasteurii* urease

Substance	Enzyme[c]	Type of Inhibition	K_i [b] ± SEM[a]
Symplocomoside (1)	J.B urease	Non-competitive	77.60 ± 1.22
	B.P urease	Non-competitive	63.47 ± 0.92
Thiourea (Standard)	J.B urease	Competitive	18.15 ± 0.77
	B.P urease	Competitive	15.38 ± 0.39

Experimental

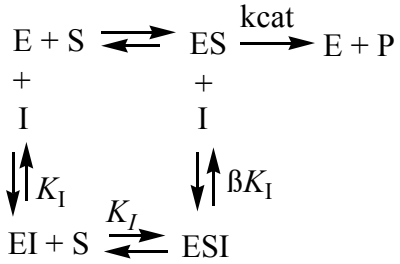
Our research group has already published the isolation and structure elucidation of symplocomoside (**1**) from *Symplocos racemosa* Roxb [11].

Urease Assay and Inhibition

Reaction mixtures comprising 25 μL of enzyme (jack bean urease and *Bacillus pasteurii* urease separately) solution and 55 μL of buffers containing urea (2-24 mM) were incubated with 5 μL of **1** at 30^{o}C for 15 min in DMSO in 96-well plates. The increased absorbance at 560 nm was measured after 10 min., using a microplate reader (Molecular Device, USA). All reactions were performed in triplicate in a final volume of 200 μL. The results (change in absorbance per min) were processed by using SoftMax Pro software (Molecular Device, USA). All the assays were performed at pH 6.8 (3 mM sodium phosphate buffer) and 7 μg of phenol red per ml as indicator. Percentage inhibitions were calculated from $100-(OD_{testwell}/OD_{control}) \times 100$. Thiourea was used as the standard inhibitor of urease.

Determination of Kinetic Parameters

The concentration of **1** that inhibited the hydrolysis of substrates (jack bean and *Bacillus pasteurii* ureases separately) by 50% (IC_{50}) was determined by monitoring the inhibition effect of various concentrations of **1** in the assay. The IC_{50} (inhibitor conc. that inhibits 50% activity of both enzymes) values were then calculated using the EZ-Fit Enzyme Kinetics program (*Perrella Scientific Inc.*, *Amherst*, USA). The interaction of **1** with jack bean and *Bacillus pasteurii* ureases can be described by the following scheme:



Scheme 1: Non-competitive inhibition.

where ES is the jack bean and *B. pasteurii* urease-urea complex and P is the product. K_I and βK_I are the inhibition constants reflecting the interactions of **1** with the free J.B. and B.P. enzymes and enzymes-urea complex [12-14].

Statistical Analysis

Graphs were plotted using GraFit program [15]. Values of the correlation coefficients, slopes, intercepts and their standard errors were obtained by the linear regression analysis using the same program. The correlation for all the lines of all graphs was found to be > 0.99. Each point in the constructed graphs represents the mean of three experiments.

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