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5-Hydroxy-1,4-naphthoquinone (juglone) and 2-hydroxy-1,4-naphthoquinone (lawsone) influence on jack bean urease activity: Elucidation of the difference in inhibition activity

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ABSTRACT

The aim of this study was elucidation of the difference in inhibition influence of 5-hydroxy-1,4-naphthoquinone (juglone) and 2-hydroxy-1,4-naphthoquinone (lawsone) on jack bean urease activity. It was found that juglone acted as a strong, time and concentration dependent inactivator of urease. On the contrary, lawsone showed an inconsiderable inhibition influence. The reactivation of juglone modified urease showed the participation of reversible and irreversible contribution in the inactivation. In the presence of an excess of DTT, urease inactivated by juglone regained 70% of its activity. The reversible inactivation was attributed to oxidation of the essential urease thiols by reactive oxygen species (ROS) realizing during reduction of juglone to seminaphthoquinone. Presence of hydrogen peroxide in the incubation system was proved by direct determination and by application of catalase. The irreversible contribution in the inhibition was assumed as an arylation of urease thiol groups by juglone. The insignificant urease inhibition by lawsone was concluded as an effect of a low hydrogen peroxide generation and lawsone resistance for reaction with protein thiols. It was found that lawsone well reacted with L-cysteine, poorly with glutathione and hardly with urease thiols. The observed sequence was arranged according the rule the more complex thiol the less susceptible for reaction with lawsone. On the other hand, juglone displayed an excellent reactivity towards both thiols and urease. Thus, this indicated a significance of a steric hindrance which appeared when the hydroxyl group changing position from 5 in juglone (5-hydroxy-1,4-naphthoquinone) to 2 in lawsone (2-hydroxy-1,4-naphthoquinone).

1. Introduction

Urease (urea amidohydrolase, EC 3.5.1.5) is an enzyme catalyzing the hydrolysis of urea: $CO(NH_2)_2 + H_2O \xrightarrow{urease} 2NH_3 + CO_2$. The enzyme has been found in plants, algae, fungi and bacteria [1–3]. The plant urease from jack bean is the homohexameric molecule (α_6). Its active site contains two nickel ions directly involved in binding of substrates and inhibitors [4,5]. Urease is a thiol rich enzyme. The total amount of thiols is equal 15 per jack bean urease subunit. However, only 6 of 15 cysteines are accessible to the thiol reagent (without denaturation of the enzyme). One of them, cysteine-592 plays critical role in the catalytic activity [6,7].

Beside the unquestionable environmental positive role of urease, some effects are damaging for human and animal health, as well as for nature. The prevention from harmful activity of the enzyme requires effective inhibitors which have been sought among numerous organic and inorganic compounds [3,8]. The specific group of examined chemicals are quinones [9–11]. Quinones have been extensively studied for their potent redox power. This ability involves them in enzymatic and nonenzymatic redox cycling with their corresponding semiquinone radicals. The side effect is the production of ROS (reactive oxygen species) such as superoxide radicals, hydrogen peroxide, and, in the presence of metal ions, hydroxyl radicals. The ROS as a strong oxidizing agents may be responsible for damage of macromolecules. Quinones also can covalently modify cellular nucleophiles such as glutathione or cysteine residues [12–16].

The quinones selected for this study, juglone and lawsone, are chemicals of the natural origin. Juglone (5-hydroxy-1,4-naphthoquinone) is a brown dye isolated from fruits, shells and leaves of walnut trees (*Juglans*). The main source of lawsone (2-hydroxy-1,4-naphthoquinone) is a henna herb *(Lawsonia inermis L.)*. The henna powder is used as a traditional pigment for dyeing hair, skin and nails. Juglone is known as an inhibitor of several enzymes [17–19]. Both naphthoquinones have been studied as the toxic factors of isolated hepatocytes [20–22]. D'Arcy Doherty and Rogers found that lawsone and juglone caused cytotoxity proceeded by

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depletion of intracellular glutathione. Juglone directly reacted with glutathione in contrast to lawsone which reactivity was thought to be enzyme-mediated [20]. Öllinger and Brunmark studied hydroxysubstituted naphthoquionones and elucidated the influence of the substituent position on the toxicity to rat hepatocytes [22]. The low toxicity of lawsone was attributed to its incapacity to undergo electrophilic addition and redox cycling (as a result of its very low one-electron reduction potential) [17]. However, the evidence of lawsone mediated redox cycling was found [23,24].

Despite a great number of investigations of juglone and lawsone cytoxicity, there is still lack of clear explanation of their activity mechanism. The inhibitors were mainly studied in biological complex systems with a large amount of interactions. System complexity generates difficulties in monitoring specific relations. Thus, the purpose of this work was study juglone and lawsone action in the system with the reduced number of by-interactions. The presented work describes the kinetics and mechanism of urease inhibition by 5-hydroxy-1,4-naphthoquinone and elucidates the lack of the inhibitory effect of 2-hydroxy-1,4-naphthoquinone. The protective effect of catalase as well as the reversibility of the inactivation were investigated. The production of hydrogen peroxide in the systems with the naphthoquinone were quantified. The role of redox cycling and thiol interaction was elucidated.

2. Materials and methods

2.1. Materials

Jack bean urease, Sigma type III, specific activity 16 U mg^{-1} solid, the total content of reducing agents 0.5 μg U^{-1}, urea (Molecular Biology Reagent), dithiothreitol (DTT), ascorbic acid, L-cysteine, glutathione, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), catalase (from bovine liver), activity 1340 U mg^{-1} solid were purchased from Sigma. 5-hydroxy-1,4-naphthoquinone (juglone) and 2-hydroxy-1,4-naphthoquinone (lawsone), were from Aldrich. Colorimetric hydrogen peroxide assay kit from Cayman Chemical Company (Ann Arbor, MI). Other chemicals were obtained from POCh, Gliwice, Poland. All reagents used were of analytical grade. Phosphate buffer 50 mM, pH 7.7, was prepared by adjusting pH of phosphoric(V) acid with NaOH. EDTA (2 mM) was added to all enzyme-containing solutions.

2.2. Standard urease activity assay

The standard assay mixture (25 cm^3) consisted of 50 mM urea in 50 mM phosphate buffer, pH 7.7 and 2 mM EDTA. The reactions were initiated by the addition of small aliquots of the enzyme-containing solution (0.5 cm^3), and the urease activity was determined by measuring ammonia concentration after a 5 min reaction. Ammonia was determined by the spectrophotometric, phenol-hypochlorite method. The absorbance was registered at 630 nm [25]. The measurements were performed at ambient temperature. The activity of uninhibited urease was accounted as the control activity of 100%.

2.3. Inactivation of urease by 5-hydroxy-1,4-naphthoquinone (juglone) and 2-hydroxy-1,4-naphthoquinone (lawsone)

The solution of urease was incubated with the solution of 5-hydroxy-1,4-naphthoquinone in the absence of urea. The incubation mixture contained 1.0 mg cm^{-3} of urease, 50 mM phosphate buffer, pH 7.7, 2 mM EDTA and juglone in the range 5–40 μM and lawsone in the range 0.25–0.5 mM. The time when the enzyme and the inhibitor were mixed was marked as zero time of incubation. After appropriate period of time, aliquots from the incubation mixture were withdrawn and transferred into the standard assay mixtures for urease residual activity determination.

2.4. Reactivation of urease inactivated by juglone

The reactivation of juglone inactivated urease was studied by using DTT. The incubation mixture contained 1.0 mg cm^{-3} urease, 20 μM juglone, 50 mM phosphate buffer, pH 7.7. After 60 min incubation DTT was added. The DTT concentration in the incubation mixture was equal to 250 μM. The activity of urease was determined before and after DTT addition. After appropriate period of time, samples of the incubation mixture were withdrawn and transferred into the standard assay mixture and urease residual activity was determined. The experiment was triply repeated.

2.5. Protection influence of catalase on urease inactivation by juglone

Catalase was incubated with urease and inactivator for 20 min. The final mixtures contained 1 mg cm^{-3} urease, 200 U cm^{-3} catalase, 50 mM phosphate buffer, pH 7.7 and inactivator: 20 and 40 μM juglone, 90 mM hydrogen peroxide, respectively. After incubations, aliquots were withdrawn from the incubation mixture and were transferred into the standard assay mixtures for urease activity determination.

2.6. Quantitative determination of hydrogen peroxide generation

Hydrogen peroxide was assayed using a colorimetric hydrogen peroxide assay kit from Cayman Chemical Company (Ann Arbor, MI). The quantitative analysis was based on Fenton reaction [26]:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\cdot} + OH^-$$
$$Fe^{3+} + XO \rightarrow Fe^{3+} - XO$$

where XO is xylenol orange which forms with Fe^{3+} a stable colored complex. Hydrogen peroxide was determined in assays contained 0.1 mM juglone and lawsone (in 50 mM phosphate buffer, pH 7.7) in the absence and presence of 0.6 and 1.2 mM DTT, respectively. The absorbance was measured at 595 nm.

2.7. DTNB determination of urease-naphthoquinone and naphthoquinone-thiol interaction

1. Urease was incubated with the naphthoquinone (lawsone and juglone) for 20 and 40 min, respectively. The incubation mixture contained: 1 mg cm^{-3} urease, 25 μM naphthoquinone, 50 mM phosphate buffer, pH 7.7, 2 mM EDTA. After incubation, the mixture urease-naphthoquinone (2.5 cm^3) was transferred to a cuvette (light path 5 cm) and mixed with 2.5 cm^3 0.15 mM DTNB (prepared in 50 mM phosphate buffer, pH 7.7). The absorbance was measured at 412 nm into continuous mode for 5 min according to Ellman's protocol [27]. The control measurements of absorbance of the used mixtures were performed: urease and DTNB-naphthoquinone in the proportions corresponding to the final reaction mixture at 412 nm into continuous mode for 5 min. The recorded control absorbances were subtracted from the absorbances recorded for the reaction mixtures: urease-DTNB-naphthoquinone.

The activity of urease before and after the incubation with naphthoquinone was tested in the standard experiment.

2. Thiol (L-cysteine and glutathione, respectively) was incubated with the naphthoquinone (juglone and lawsone, respectively) for 10 min. The incubation mixture contained: 0.05 mM thiol, 25 μM naphthoquinone, 50 mM phosphate buffer, pH 7.7. The continuation of the procedure is described above at the point 1. The control measurements of absorbance of the used mixtures were performed