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Inhibition of jack bean urease by *p*-benzoquinone: elucidation of the role of thiols and reversibility of the process

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Abstract

p-Benzoquinone (pBQ) was studied as an inhibitor of jack bean urease in 20 mM phosphate buffer, pH 7.0, 1 mM EDTA, 25 °C. The inhibition was carried out by the use of a preincubation procedure in the absence of substrate. The influence of the inhibitor concentration and the preincubation time on the enzyme activity was elucidated. It was found that increase in pBQ concentration resulted in a linear decrease of urease activity. The dependence of the enzyme activity on the preincubation time showed that the rate of inhibition rapidly decreased at the beginning of the process in order to achieve the constant value. The inhibition became time independent in the studied time range. This observation is characteristic of a slow binding mechanism of inhibition. The protective experiment proved that the urease active site is involved in the binding of pBQ. High effectiveness of thiol protectors against pBQ inhibition indicates the strategic role of the active site sulfhydryl group in the blocking process. There were two methods used for reactivation of pBQ-inhibited urease. The dilution of the urease-pBQ complex in urea solution did not result in a regain of enzyme activity. Alternatively, the addition of dithiothreitol into the urease-pBQ mixture caused the instant and efficient reactivation of the enzyme. The experiments showed that the nature of the urease-pBQ complex is irreversible but the application of a specific thiol reagent can release the active enzyme from the complex.

Keywords: *Urease, inhibition, quinone,* p-*benzoquinone*

Introduction

Ureases (urea amidohydrolase, EC 3.5.1.5) are nickel metalloenzymes that catalyze the hydrolysis of urea: $CO(NH_2)_2 + H_2O \rightarrow 2NH_3 + CO_2$ and have been isolated from many organisms including plants, bacteria and fungi. The bacterial ureases are multimer-complexes while the plant and fungal ureases are homo-oligomeric proteins. In spite of differences in number and structure of subunits all known ureases show high similarity in amino-acid sequence of the active sites. The best-characterised urease is that from jack bean. Jack bean urease exists as a homotrimer able to aggregate to a homohexamer. Each catalytic subunit contains the active site with two nickel ions. This metallocenter is directly involved in binding of substrates and inhibitors [1–5]. Ureases are thiol-rich molecules. The number and importance of thiol residues has been determined by the use of thiol reactive agents: 2,2′-dithiodipyridine, 5,5′-dithiobis(2-nitrobenzoic acid), N-ethylmaleimide. The studies proved that jack bean urease posseses six thiol groups essential for enzyme activity; among them is one unique thiol residue (cysteine 592), the modification of which results in the total abolishion of catalytic activity [1,6–8].

As a result of urease activity, urea, a very stable molecule, can be used by organisms as the nitrogen source. In plants, urease also acts as a defence protein in systemic nitrogen transport pathways. On the other hand, the products of urea hydrolysis causes a pH increase, the major reason for the negative effects of

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urease action for human and animal health, as well for the environment. Bacterial ureases have been shown to be important determinants in pathogenesis in human and animals (e.g. infection stones, hepatic coma, gastric and peptic ulcers). In agriculture, high urease activity causes environmental and economic problems. Too fast urea hydrolysis decreases efficiency of fertiliser use, moreover the abnormal quantity of ammonia induces damage to germinating seeds, seedlings and young plants. The most successful approach to solve these problems is a potent urease inhibitor. Urease inhibitors have been searched among various organic and inorganic compounds [4,9,10]. This research have been made more effective since determination of the high-resolution X-ray structures of native and inhibited ureases from *Klebsiella aerogenes* and *Bacillus pasteurii* [11–13]. The promising group of the tested chemicals are quinones, which are compounds of wide occurrence in nature. They are known for their bacteriostatic and fungicidal action as well as for their inhibitory influence on certain enzymes, such as carboxylase and urease. The substituent group on the quinone largely influences the quinone inhibition effectiveness. It has been shown that halogen-substituted derivatives are usually potent urease inhibitors, the most efficient chloranil (tetrachloro-*p*-benzoquinone) and dichlone (2,3-dichloro-*p*-naphthoquinone) are used as fungicides [14–16].

The aim of the present work was to study the inhibitory effect of *p*-benzoquinone (pBQ) on jack bean urease. The inhibition was studied by monitoring the reaction initiated by addition of substrate after preincubation of urease with the inhibitor (the preincubation technique). Moreover, thiols and inorganic inhibitors of urease were examined as protectors against pBQ-inhibition of urease. The inhibited urease reactivation was studied using multi-dilution method and dithiothreitol application. The studies, based on the reaction with thiols have elucidated the role of the urease active-site sulfhydryl group in the inhibition by pBQ. The report completes previous studies [17].

Materials

Jack bean urease, Sigma type III of specific activity 16 units/mg protein was used. One unit is the amount of enzyme that liberates 1.0 μmol of NH_3 from urea per min at pH 7.0 and 25 °C. Urea (Molecular Biology Reagent), L,D-dithiothreitol (DTT), glutathione (Glu), L-cysteine (L-Cys), were purchased from Sigma and the inhibitor *p*-benzoquinone (pBQ) from Aldrich. Other chemicals were obtained from POCh, Gliwice, Poland. All reagents used were of analytical grade.

Methods

Ammonia determination

The hydrolysis of urea catalyzed by jack bean urease: $CO(NH_2)_2 + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2$, was monitored by measuring the ammonia concentration. The amount of ammonia was determined by the spectrophotometric, phenol-hypochlorite method, the absorbance was measured at 630 nm [18].

Enzyme activity measurement

A concentrated solution of urease was preincubated with a concentrated solution of inhibitor in the absence of substrate. The preincubation solution contained 1.0 mg cm^{-3} of urease, 20 mM phosphate buffer, pH 7.0, 1 mM EDTA and different pBQ concentrations (3, 5, 6, 8, 10 μM). The time when the enzyme and the inhibitor were mixed was taken as zero time of preincubation. After appropriate periods of time, aliquots were withdrawn from the preincubation solution and diluted 50-fold into the reaction mixtures (50 mM urea, 20 mM phosphate buffer, pH 7.0, 1 mM EDTA). After 5 min a sample of the reaction mixture was withdrawn and the amount of ammonia was determined.

The amount of ammonia released in the reaction mixture for 5 min after addition of uninhibited urease was considered as a control activity of 100%. All measured enzyme activities were normalized to the control activity.

Protective experiment

In the protective experiment, all the preincubation mixtures contained 1.0 mg cm^{-3} urease, 20 mM phosphate buffer, pH 7.0, 1 mM EDTA, 7.5 μM pBQ and the protector: 12.5 mM dithiothreitol, 12.5 mM L-cysteine, 2.5 mM glutathione, 12.5 mM boric acid (BA), 12.5 mM sodium fluoride, respectively.

The components of the preincubation mixture were mixed according to two orders of mixing:

a) urease was added to the mixture after a 20 min contact of pBQ with the protector.
b) pBQ was added to mixture after a 20 min contact of urease with the protector.

The preincubation mixtures containing all components were preincubated further for 20 min. Next, a sample of the preincubation mixture was withdrawn and diluted 50-fold into the reaction mixture (50 mM urea, 20 mM phosphate buffer, pH 7.0, 1 mM EDTA). The amount of ammonia released for 5 min was determined by the phenol-hypochlorite method.

Reactivation of pBQ inhibited urease

The reactivation of inhibited urease was studied in two ways; using DTT and by multidilution in the reaction mixture containing urea.

1) The preincubation mixture contained 1.0 mg cm^{-3} urease, 20 mM phosphate buffer, pH 7.0, 1 mM EDTA, 10 μM pBQ. After a 20 min preincubation DTT was added (DTT concentration in the preincubation mixture was equal to 5 mM). The activity of urease was determined before and after the addition of DTT.
2) The samples of preincubation mixture (1.0 mg cm^{-3} urease, 20 mM phosphate buffer, pH 7.0, 1 mM EDTA, 10 pBQ) after a 10 and 20 min preincubation, respectively, were diluted 50-fold into the reaction mixture: 50 mM urea, 20 mM phosphate buffer, pH 7.0, 1 mM EDTA.

After appropriate periods of time, aliquots were withdrawn and the amount of ammonia was determined.

Results and discussion

Enzyme activity measurement

The obtained results of enzyme activity measurement *versus* preincubation time are presented in Figure 1. Urease was preincubated with pBQ in the range of 3–10 μM. It was shown that upon increasing the time of preincubation a decrease in urease activity was observed. The loss of activity was rapid in the beginning until the equilibrium between urease E, the inhibitor I and urease-inhibitor complexes EI and EI* (E + I ⇔ EI ⇔ EI*) was achieved. The final recorded state characterised by the constant activity of urease relates to the equilibrium. The obtained relationship of enzyme activity versus time of preincubation are characteristic of slow binding

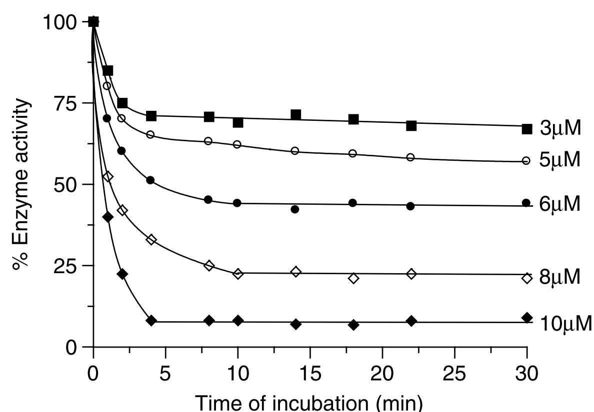

Figure 1. Dependence of % urease activity vs preincubation time with pBQ. Concentration of pBQ [μM] is numerically given.

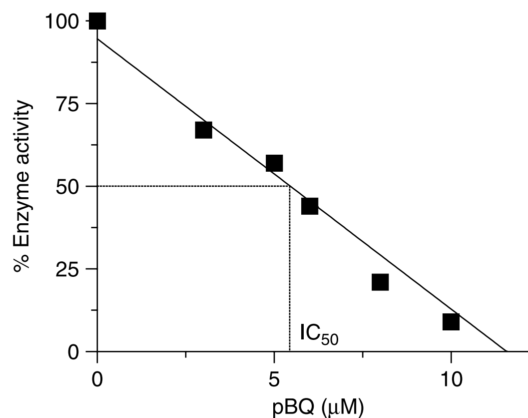

Figure 2. Dependence of % urease activity vs concentration of pBQ.

inhibition [19]. This is consistent with our previous studies using techniques without preincubation (enzymatic reaction initiated by the addition of the enzyme and the progress curves monitored). The term enzyme activity applied to slow binding inhibition means the total urease activity in the free form of the enzyme as well the enzyme bound in the complexes EI and EI*.

Figure 2 presents enzyme activity as a function of pBQ concentration. The linear function for this relation is a good enough approximation ($R^2 = 0.96$). The obtained IC_{50} value was equal to 5.1 μM. The inhibitory pBQ concentration range towards urease indicated that pBQ is a potent inhibitor and can be classified among the strongest urease inhibitors such as phenylphosphorodiamidate [10] and Hg(II) ions [20]. Ashiralieva et al. [16] classified pBQ as a non-competitive urease inhibitor. The slow binding inhibition can be misinterpreted as a non-competitive type if the inhibition constant is determined by applying the initial reaction rates method. This method is useless for slow binding inhibition studies because the slow binding effect is revealed only after sufficient time elapses for the enzyme-inhibitor interaction. Our previous thorough investigation by the progress curves analysis [17] and preincubation studies presented in this paper proved the slow binding model of urease inactivation by pBQ.

Protective experiment

It was shown that the presence of monothiols, L-cysteine and glutathione, as well as dithiol, L,D-dithiothreitol, in the preincubation mixture prevented urease being inhibited by pBQ (Figure 3). No significant influence of the order of components addition on the protection effect was observed. The enzyme retained more than 70% of its control activity

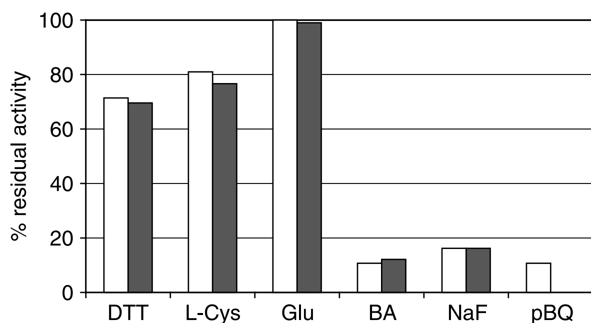

Figure 3. Protective effects of 12.5 mM L,D-dithiothreitol (DTT), 12.5 mM L-cysteine (L-Cys), 2.5 mM glutathione (Glu), 12.5 mM boric acid (BA) and 12.5 mM sodium fluoride against the urease inhibition by 7.5 μM pBQ. White and black columns relate to the experiment where the last added agent was pBQ or urease, respectively (details in Experimental section). The percent of enzyme activity in the presence of pBQ without the protector is given for comparison.

after preincubation with pBQ and thiol-protector, while the preincubation without the thiol-protector resulted in a decrease of activity up to almost 10%. Glutathione was found to be the most potent protector among the tested compounds. The protection ability of sodium fluoride (a competitive slow binding urease inhibitor [21]) and boric acid (a classical competitive urease inhibitor [22]) was insignificant. Sodium fluoride and boric acid inhibit urease by interaction with active site nickel ions [21,22] while thiols reacts with sulfhydryl groups. The interaction target in the enzyme for these protectors is different. The experiment suggested that the protection occurs if the protector and the inhibitor compete for the same strategic group in enzymatic activity.

The mechanisms of quinone cytotoxic effects are thought to be quite complex. However, two main mechanisms have been elucidated. Cellular damage can occur through alkylation of crucial proteins. Alternatively, quinones as highly redox active molecules can cause loss of protein thiols that leads to alteration in protein structure and function [23]. The result of the protective experiment indicated the modification of the active site cysteine (residue 592) as a reason for pBQ inhibition of urease.

Makinen et al. [24] studied inactivation of bacterial collagenase by tetrachloro-*p*-benzoquinone. They suggested that inactivation was due to reaction with a tyrosine active site residue. The jack bean urease amino acid sequence [7] in the active site region (residues 479–607) contains 4 tyrosyl residues but none of them is known as essential for urease activity. The reaction of pBQ with that residue(s) is less probable as a reason for the blocking of urease activity.

Reactivation of inhibited urease

The reactivation of pBQ-inhibited urease was studied in two ways. In the first approach, DTT was applied. The method with DTT was thought to be promising due to the reactivity of quinones towards thiols and the obtained results (Figure 4A) confirmed this presumption. pBQ-inhibited urease showed a rapid and significant recovery of activity after DTT application. The activity increased from 10% up to 75%. The experiment indicated on a better affinity of quinones towards DTT than the thiol group in urease.

The next method uses a multidilution as a means of enzyme release from the enzyme-inhibitor complex. pBQ was preincubated with the enzyme to establish the equilibrium: $E + I \Leftrightarrow EI \Leftrightarrow EI^{\star}$, the preincubation time being selected from the enzyme activity measurement (Figure 1). The dilution of the preincubation mixture in the solution containing the substrate initiated the enzymatic reaction. The progress curves obtained after dilution of the preincubation mixture are presented in Figure 4B. The curves displayed a linear increase in ammonia concentration which indicated constant activity/amount of urease in the system and lack of enzyme release from the urease-inhibitor complex. The experiment pointed out the irreversibility of the urease-pBQ complex; the release of

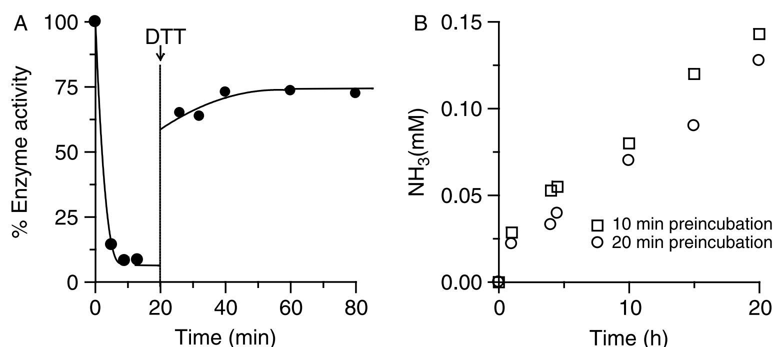

Figure 4. Reactivation progress curves of inhibited urease by pBQ. A) reactivation by addition of DTT B) reactivation by 50-fold dilution in 50 mM urea.

active enzyme from the complex needs a chemical approach.

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