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Irreversible Inhibition of Jack Bean Urease by Pyrocatechol

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Pyrocatechol was studied as an inhibitor of jack bean urease in 20 mM phosphate buffer, pH 7.0, 25°C. The inhibition was monitored by an incubation procedure in the absence of substrate and reaction progress studies in the presence of substrate. It was found that pyrocatechol acted as a time- and concentration dependent irreversible inactivator of urease. The dependence of the residual activity of urease on the incubation time showed that the rate of inhibition increased with time until there was total loss of enzyme activity. The inactivation process followed a non-pseudo-first order reaction. The obtained reaction progress curves were found to be time-dependent. The plots showed that the rate of the enzyme reaction in the final stages reached zero. From protection experiments it appeared that thiol-compounds such as L-cysteine, 2-mercaptoethanol and dithiothreitol prevented urease from pyrocatechol inactivation as well as the substrate, urea, and the competitive inhibitor boric acid. These results proved that the urease active site was involved in the pyrocatechol inactivation.

Keywords: Urease; Inactivation; Pyrocatechol; Jack bean

INTRODUCTION

Urease (urea amidohydrolase, EC 3.5.1.5) catalyzes the hydrolysis of urea: $CO(NH_2)_2 + H_2O \rightarrow 2NH_3 + CO_2$. The enzyme metallocenter contains two nickel ions, which are ~3.5 Å apart and liganded by three and four protein atoms, respectively. This metallocenter is directly involved in binding of substrates and inhibitors.[1–4]

Urease is thiol rich enzyme. Urease jack bean contains in total 15 cysteine residues per subunit. One of them, cysteine-592, has been proved to be essential for enzymatic activity.[4,5] Some inhibitors reacting with thiol groups have been recognised as compounds that inhibit urease with total inactivation of the enzyme. Among that class of urease inhibitors are reagents which specifically react with sulfhydryl groups: the alkylating agents N-methylmaleimide, iodoacetamide, iodoacetic acid and disulphide reagents such as 5,5′-dithiobis(2-nitrobenzoic acid). These reagents inactivate urease by a pseudo-first order process.[6–8] The unique, biphasic process of urease inactivation is observed with Ag(I) that also blocks the essential thiol groups of the enzyme.[9]

Significant quantities of urea are constantly released into the environment through biological actions. As a result of enzymatic catalysis urea is generally shortlived. Urease allows the organism to use urea as the nitrogen source and moreover, urease participates in systemic nitrogen transport pathways in plants. On the other hand, the common use of urea as nitrogen fertilizer requires reduction of the catalytic action of urease in soil. That has caused an intensive search for an effective, agriculturally applicable inhibitor. A comparison of tested compounds has shown that the organic urease inhibitors are more potent as compared to inorganic inhibitors. Which has directed studies towards benzoquinones, hydroquinones and their derivatives.[10–14] Bremner and Douglas found pyrocatechol as one of the most promising inhibitors of soil urease.[14]

In this paper the inhibitory influence of pyrocatechol on jack bean urease has been studied. The protective effects of thiols, competitive urease inhibitors and substrate were studied and the mechanism and kinetics of inactivation were examined.

MATERIALS AND METHODS

Materials

The jack bean urease, Sigma type III of specific activity 22 units/mg protein, was used. One unit is

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the amount of enzyme that liberates 1.0 μmol of NH_3 from urea per minute at pH 7.0 and 25°C. Urea (Molecular Biology Reagent), Hepes, L-cysteine (L-cys), 2-mercaptoethanol (2-ME) and dithiothreitol (DTT) were purchased from Sigma and 1,4-benzoquinone (BQ) and the inhibitor pyrocatechol (1,2-dihydroxybenzene, DHB) from Fluka Chemika. Other chemicals were obtained from POCh, Gliwice, Poland. All reagents used were of analytical grade.

Inhibition of Urease

The hydrolysis of urea catalyzed by jack bean urease in the presence of pyrocatechol was studied in 20 mM phosphate buffer, pH 7.0, 1 mM EDTA at 25°C. The reaction was monitored by measuring the ammonia concentration. Liberated by the phenol-hypochlorite colorimetric method.[15] The effect of pyrocatechol on the phenol-hypochlorite method was tested and only a little interference was found. The correction factor was determined and taken into account in all calculations. Two procedures for the inhibition studies were applied: the incubation procedure and progress-curves studies.

Incubation Procedure

In the incubation procedure a concentrated solution of urease was incubated with a concentrated solution of pyrocatechol in the absence of substrate.

The incubation solution contained 0.5 mg cm^{-3} of urease and different concentrations of pyrocatechol (1.25, 2.0, 2.5 mM). The time when the enzyme and the inhibitor were mixed was taken as zero time of incubation. After appropriate periods of time, aliquots were withdrawn from the incubation solution and diluted 50-fold into the reaction mixture (50 mM urea, 1 mM EDTA, 20 mM phosphate buffer, pH 7.0). 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 over 5 min after addition of uninhibited urease was counted as a control activity of 100%.

Progress-curves Studies

Progress curves were obtained for the reactions initiated by the addition of enzyme into the reaction mixtures containing different concentrations of DHB (0.05, 0.06, 0.07, 0.1 mM) and 50 mM urea, 1 mM EDTA, 20 mM phosphate buffer, pH 7.0. The concentration of urease in the reaction mixture was 0.01 mg cm^{-3}. After appropriate periods of time, aliquots were withdrawn from the reaction mixture and the amount of ammonia was determined.

Protection Experiment

In the protection experiment, the incubation mixture contained 0.5 mg cm^{-3} urease, 20 mM phosphate buffer, pH 7.0, 1mM EDTA, 2.5 mM pyrocatechol and 12.5 mM protector. A ten fold higher concentration of phosphate buffer (200 mM) was used when urease was incubated with urea as a protector. The higher concentration of buffer retained a neutral pH when ammonia was released during incubation. After a 20 min incubation a sample of the incubation mixture was withdrawn and diluted 50-fold into the reaction mixture (50 mM urea, 1 mM EDTA, 20 mM phosphate buffer). After 5 min the amount of ammonia was determined.

The time-dependence of protection of urease by boric acid and 1,4-benzoquinone against inactivation by pyrocatechol was monitored under identical conditions to those described above. Aliquots were withdrawn from the incubation mixture at different time intervals and the residual activity was measured. The experiment was repeated in the absence of pyrocatechol and the presence of the protector.

Theory and Equations

An enzyme inactivator is a compound that irreversibly inhibits the enzyme due to formation of a stable covalent bond(s) between the inhibitor and essential functional groups of enzyme. The inactivation is progressive with time eventually reaching complete inhibition of the enzyme.[16] The anticipated reaction scheme for the inactivation of enzyme E by inhibitor I is:[17]

$$E + I \underset{k_2}{\overset{k_1}{\Leftrightarrow}} EI \xrightarrow{k_3} EI^* \tag{1}$$

The total amount of enzyme is given by:

$$E^o = E + EI + EI^* = \varepsilon + EI^* \tag{2}$$

The dissociation constant for the reversible step of the enzyme-inhibitor interaction can be written as:

$$K_i = k_2/k_1 = I \cdot E/EI \tag{3}$$

The rate of formation of EI* is expressed by:

$$-\frac{d\varepsilon}{dt} = k_3 EI \tag{4}$$

Solution of Equation (4) has the following form:

$$\ln \frac{\varepsilon}{E^o} = -\frac{k_3}{1 + K_i/I} t \tag{5}$$

where ε/E^o defines the residual activity of the enzyme at time t.

$$\text{If } I \gg E_o \text{ and } k_{app} = \frac{k_3}{1 + K_i/I} \tag{6}$$

then the reciprocal of k_{app} is:

$$\frac{1}{k_{app}} = \frac{1}{k_3} + \frac{K_i}{k_3}\frac{1}{I} \tag{7}$$

The data plotted in accordance with Equation (7) leads to determination of K_i and the inactivation constant k_{inact}, equal to $1/k_3$. If $I \ll K_i$, $k_{app} = (k_3/K_i)^*I$ and k_3' can be set instead of k_3/K_i. This means that the kinetics are not distinguishable from a simple bimolecular mechanism and the data plotted in accordance with Equation (7) gives a straight line passing through the origin.[16]

RESULTS AND DISCUSSION

Analysis of the Reaction Progress Curves

The results of the analytical studies of pyrocatechol-inhibited enzymatic hydrolysis of urea for different concentrations of the inhibitor are presented in Figure 1. The curves demonstrate a time-dependent character for the inhibition. The velocity of the reaction was initially fast and then slowed down, finally leading to complete inhibition. This effect was demonstrated more clearly, the higher the concentration of pyrocatechol present in the system.

Incubation of Urease with Pyrocatechol

The data obtained from the incubation studies are presented in Figure 2. Urease was incubated with 1.25, 2.0, 2.5 mM pyrocatechol, respectively.

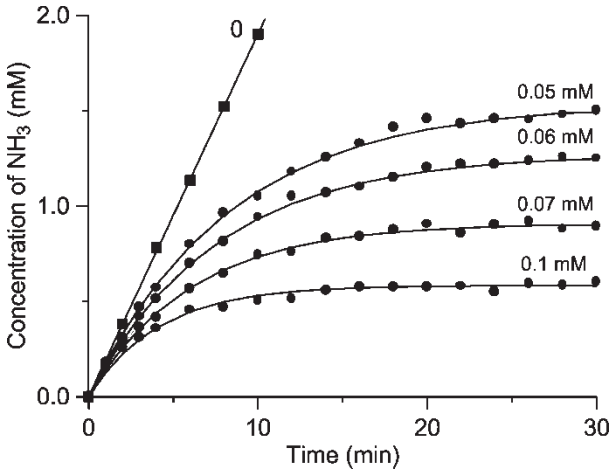

FIGURE 1 Reaction progress curves for the urease-catalyzed hydrolysis of urea in the presence of pyrocatechol. Concentration of pyrocatechol [mM] is numerically given.

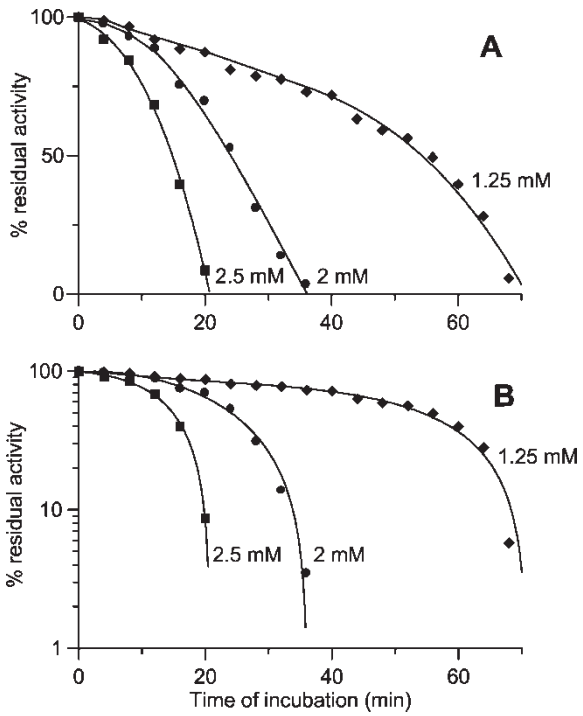

FIGURE 2 (A) Dependence of residual activity of urease *vs* incubation time with 1.25, 2.0 and 2.5 mM pyrocatechol. (B) Replot of results on a semi-logarithmic scale.

The concentration of pyrocatechol was in large excess relative to urease ($I \gg E_o$). It was shown that increasing the time of incubation resulted in a decrease of urease activity with an eventual total loss of catalytic activity. Moreover, an increase in the concentration of the inhibitor produced an increase in the rate of inhibition. These results indicated that the inhibition of urease by pyrocatechol was time- and concentration dependent. In order to determine if pyrocatechol interacted with urease irreversibly, the enzyme was incubated with the inhibitor until complete inhibition was reached. Then, the pyrocatechol-modified urease was dialysed at 4°C, for 24 h in 20 mM phosphate buffer, 1 mM EDTA and the enzymatic activity was measured. Urease did not regain its activity, which proved the irreversibility of the urease-pyrocatechol interaction.

Transformation of the incubation data onto a semi-logarithmic scale, according to Equation (5) (Figure 2 B), showed a clear downward deviation from linearity. This case is observed when there is an increase in the rate of inactivation with time. The obtained non-linear kinetics replots indicated non-pseudo-first order kinetics. The increase in the rate of inactivation suggested that the initial compound incubated with the enzyme was converted to another compound, which was the actual inactivator of the enzyme; when the concentration of the actual inactivator increased with the time of incubation, the rate of inactivation also increased, until it reached a maximum saturation

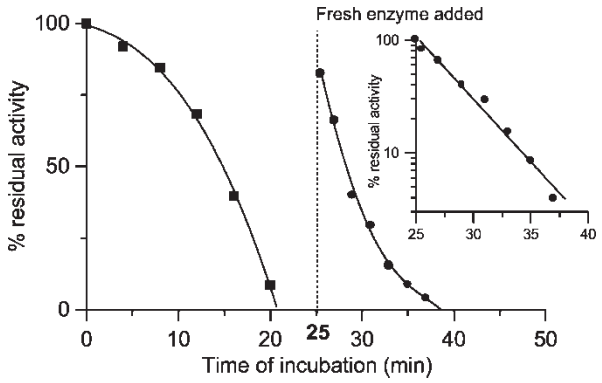

FIGURE 3 Dependence of residual activity of urease *vs* incubation time with 2.5 mM pyrocatechol. A fresh amount of urease, equal to that at zero time of incubation, was added in the twenty fifth minute of the process (Inset: replot on a semi-logarithmic scale).

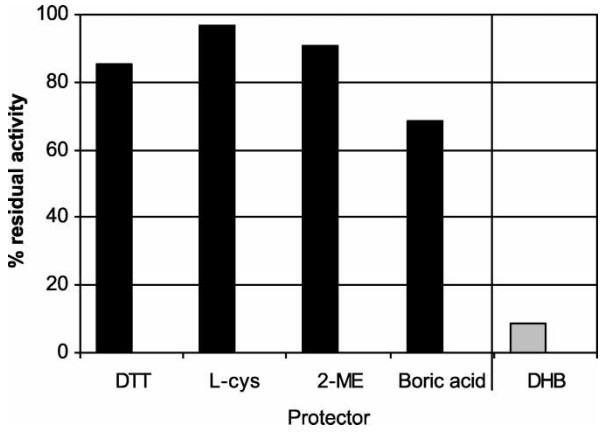

FIGURE 4 Protective effects of dithiothreitol (DTT), L-cysteine (L-cys), 2-mercaptoethanol (2-ME) and boric acid on urease inhibition by pyrocatechol, relative to the control activity. The percent of the residual activity of urease in the presence of pyrocatechol (DHB) without the protector is given for comparison. Concentration of protector and pyrocatechol was equal to 12.5 mM and 2.5 mM, respectively.

rate. After completion of the enzyme inactivation the reaction mixture should still be saturated with the actual inactivation species so that a fresh aliquot of enzyme added to the reaction mixture would be inactivated at the maximum rate.[16] This effect was observed in the studied system. After the addition of a fresh aliquot of urease into the system, where the inactivation of the enzyme had been already completed, the process of inactivation commenced immediately at the maximum rate (Figure 3). This result assumes that there was conversion of pyrocatechol into the actual inactivator according to the scheme (Equation 8):

$$\mathrm{DHB} + \mathrm{urease} \Leftrightarrow \mathrm{DHB} - \mathrm{urease} \rightarrow$$

$$\mathrm{DHB}^* + \mathrm{urease} \rightarrow \mathrm{DHB}^* - \mathrm{urease} \qquad (8)$$

Moreover, the inactivation of urease by that new species was a pseudo-first order process as demonstrated by the straight line produced by a replot of the data on a semi-logarithmic scale (Figure 3, inset).

If the actual inhibitor released from the enzyme is a radical species or any electrophile, a trapping agent should prevent the inactivation.[16] It was shown that the presence of the monothiols L-cysteine and 2-mercaptoethanol as well the dithiol, dithiothreitol, in the incubation mixture prevented inactivation of urease by pyrocatechol (Figure 4). The enzyme retained more than 80% of its control activity after a 20 min incubation, while the inactivation without the thiol-protector resulted almost in total inactivation.

The simplest explanation of the transformation of pyrocatechol might be an oxidation of pyrocatechol into o-quinone, a more effective inhibitor of urease than pyrocatechol. Pyrocatechol as a nickel chelator ($pK = 8.77$[18]) could be responsible for the initial, slow period of the inactivation due to a complexing process with nickel ions at the urease active site. The increasing amount of the formed o-quinone can explain the increasing rate of urease inactivation. Moreover, o-quinones are highly reactive towards nucleophiles which explains the thiol-prevention effect.

Protective Experiment-time Dependence

The time-dependence of protection against inactivation of urease by pyrocatechol was studied. Three classes of protectors were used: the competitive inhibitor boric acid, [19] the competitive, slow-binding inhibitor BQ[13] and the substrate urea. All the protectors used interact with urease at the active site so that the protective effect should indicate if the pyrocatechol inhibition occurs at or outside the active site. The inactivation mixture contained urease with 2.5 mM DHB and an excess of the respective protector (12.5 mM). In the separate experiments the inhibition influence of the protector on urease was measured under identical conditions. The results are shown in Figure 5. The studied competitive, slow binding inhibitor BQ showed no protective effect in contrast to the classical competitive inhibitor, boric acid. Since the urease active site is involved in the inhibition of both inhibitors used they would be expected to show the same protective effect. The observation that only boric acid demonstrated the protective effect indicated that the slow-binding inhibitor was unable to protect the enzyme. This effect was probably caused by a relatively long equilibration time. The equilibrium in the slow binding inhibition between enzyme, inhibitor and enzyme-inhibitor complex was established slowly, therefore the unbound enzyme could take part in the conversion DHB into DHB*. DHB* was suggested

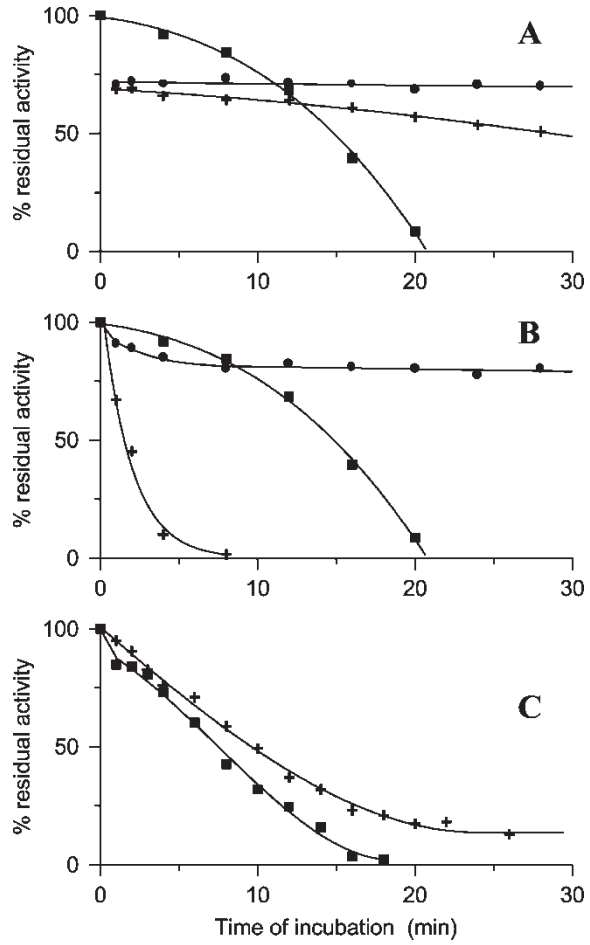

FIGURE 5 Time-dependence of protective effects of (A) boric acid, (B) benzoquinone (BQ) and (C) urea on the inhibition of urease by pyrocatechol (DHB). Concentration of protector and pyrocatechol was equal to 12.5 mM and 2.5 mM, respectively. (■) The progress of urease inactivation by DHB. (●) The progress of urease inhibition by protector. (+) The progress of urease inactivation by DHB in the presence of protector.

to inhibit urease strongly and irreversibly. Therefore the competition between protector and inhibitor was in fact the competition between protector and inactivator. In the system with boric acid the equilibrium between a classical competitive inhibitor (boric acid), inhibitor DHB and urease was established sufficiently quickly to neglect the amount of DHB*. Such a mechanism indicates that the inhibition of urease by DHB occurred at the active site of urease.

When urease was inactivated by pyrocatechol in the presence of BQ, the activity of the enzyme quickly decreased to zero, much faster than in the presence of pyrocatechol alone. This effect might be interpreted as a positive synergetic phenomenon.

The studies on substrate protection showed for the assumed mechanism that the urease active site was directly involved in the inactivation process. Such an experiment required different conditions of incubation since the ammonia released during incubation of urease with urea had to be controlled by using a high buffer concentration. The high concentration of buffer resulted in a decrease in urease activity,[20] which was expressed by a different course for the time-dependence of urease inactivation by pyrocatechol (Figure 5C). The presence of urea in the incubation mixture retained approximately 20% of the control urease activity after 20 minutes, while the urease was totally inactivated by pyrocatechol in the absence of substrate. This fact confirmed that the inactivation of urease by pyrocatechol was at the urease active site. On the other hand, a more complicated character of inactivation as well as the occurrence of two or more simultaneous inactivation mechanisms cannot be excluded.

Acknowledgement

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