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A SIMPLE NOVEL METHOD FOR STUDYING THE COMBINED INHIBITORY EFFECTS OF ETHYLUREA AND *N,N*-DIMETHYLUREA ON JACK BEAN UREASE

A.A. SABOURY[1] and A.A. MOOSAVI-MOVAHEDI[2,*]

[1]*Department of Chemistry, University of Tarbiat-Modares, Tehran, Iran*
[2]*Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran*

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The kinetics of the inhibition of jack bean urease in the presence of ethylurea and *N,N*-dimethylurea was studied at pH = 7.0. Both inhibitors were competitive inhibitors. A simple novel method was used for determining the dissociation constants (K_i) values for the inhibition which were 26 and 28 mM respectively.

Keywords: Urease; combined inhibitory effects; graphical method.

INTRODUCTION

Jack bean urease (urea amidohydrolase; E.C. 3.5.1.5) was the first enzyme crystallized[1] and the first to be shown to include nickel.[2] Urease catalyzes the hydrolysis of urea to carbonic acid and two molecules of ammonia.[3]

The relative mass of the urease subunit calculated from the amino acid sequence (excluding the two nickel ions per subunit) is 90,770.[4] Since jack bean urease has six identical subunits[5], the relative molecular mass of the hexamer urease molecule is 545,340 (including 12 nickel ions).[4] This protein consists of a single kind of polypeptide chain containing 840 amino acids residue.[4]

The specificity of urease was believed to be absolute[6] until Fishbein *et al.*[7,8] reported that *N*-hydroxyurea was a substrate. Derivatives of urea which are substrates for urease include *N*-hydroxyurea[7–9], *N,N*-dihydroxyurea[10,11], semicarbazide[12]

*Correspondence.

and *N*-methylurea.[13] Formamid[14] and acetamide[13] are other examples of substrates for urease which are not substituted ureas.

Despite extensive investigation the detailed mechanism of urease is not established and the structure of the metallocenter is unknown. Zerner *et al.*[13] have proposed a model for jack bean urease catalysis in which one nickel coordinates the oxygen atom of urea, polarizing the carbonyl group, and a second nickel coordinates hydroxide ion, the catalytic nucleophile.

The study of urease inhibitors may have medical or agronomic significance, as well as providing insight into the urease catalytic mechanism.[15]

Here, we study the combined inhibitory of ethylurea (I) and (N,N) dimethylurea (X) on jack bean urease. The values for the dissociation constants of inhibitors from the enzyme-inhibitors complexes have been determined by using a simple novel graphical method.

MATERIALS AND METHODS

Jack bean urease, L-glutamic dehydrogenase (GLDH), β-nicotinamide adenine dinucleotide, reduced form (NADH), α-ketoglutaric acid (α-KG), adenosine 5′-diphosphate (ADP), tris-base and ethylendiaminetetraacetic acid (EDTA) were obtained from Sigma Chemical Co. Urea, ethylurea and *N*,*N*-dimethylurea were obtained from Aldrich.

Solution were made in double-distilled water. Tris-base solution with 30 mM concentration, pH = 7.0, was used as a buffer.

Assay Method

The hydrolysis of urea was measured by coupling ammonia production to a GLDH reaction:[16]

$$\text{Urea} + H_2O + 2H^+ \xrightarrow{\text{urease}} 2NH_4^+ + CO_2$$

$$2NH_4^+ + 2\alpha\text{-KG} + 2\text{NADH} \xrightarrow{\text{GLDH}} 2\text{Glutamate} + 2\text{NAD}^+ 2H_2O \qquad (1)$$

The decrease in absorbance at wavelength of 340 nm(ΔA_{340}) is related to the decrease of NADH concentration and the rate of reaction (V) is given by:[16]

$$V = \frac{(709.4/17.0)}{(\text{mg urease/ml reaction mixture})} \cdot \frac{\Delta A_{340}/\text{min}}{6.22} \qquad (2)$$

where 709.4 and 17.0 are the relative molecular masses of NADH and NH_3, respectively. The micromolar absorptivity of NADH is 6.22, when the length of the radiation path is 1 cm. The concentration of urease is taken as 0.025 mg/ml^{-1} of the reaction mixture. The details for the assay method are described in the Worthington enzyme manual.[16]

A recording spectrophotometer, UV-3100 Shimadzu model, was used for recording the rate of absorbance changes. The instrument is equipped with a thermostatted cell compartment.

RESULTS AND DISCUSSION

The competitive inhibition of urease (E) with ethylurea (I) and *N,N*-dimethylurea (X) is as follows:

$$\begin{array}{l} \quad EX \\ K_X \;\| \\ \quad X \\ \quad + \\ E + S \underset{}{\overset{K_S}{\rightleftharpoons}} ES \xrightarrow{K_{cat}} E + P \\ \quad + \\ \quad I \\ K_I \;\| \\ \quad EI \end{array} \tag{3}$$

where S and P are substrate and product, respectively and

$$K_S = [S][E] / [ES], \quad K_I = [I][E] / [EI], \quad K_X = [X][E] / [EX]$$

The steady-state approach[17,18] yields:

$$\frac{V}{V_{max}} = \frac{[S]}{K'_m + [S]} \tag{4}$$

$$K'_m = K_m \left(1 + \frac{[I]}{K_I} + \frac{[X]}{K_X}\right) \tag{5}$$

where V ,V_{max} and K_m are velocity, maximum velocity and Michaelis constant, respectively.

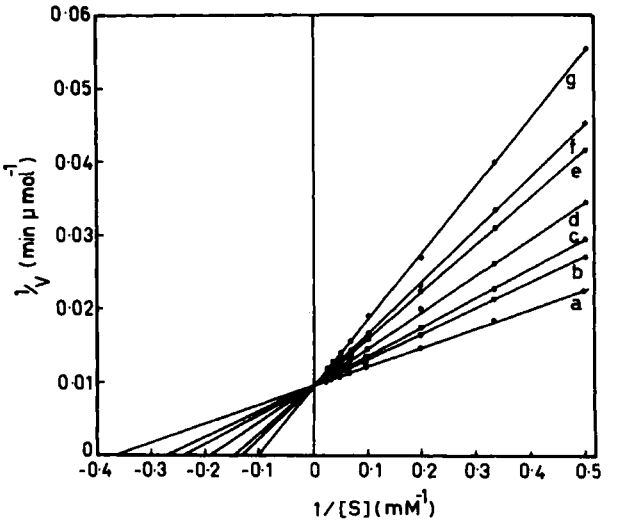

FIGURE 1 Double reciprocal Lineweaver-Burk plots for kinetics of jack bean urease at pH = 7.0 (Tris; 30 mM) and T = 27°C in different fixed concentrations of I and X: [I] = [X] = 0 mM (a), [I] = [X] = 5 mM (b), [I] = 10 mM and [X] = 5 mM (c), [I] = 20 mM and [X] = 5 mM (d), [I] = 30 mM and [X] = 10 mM (e), [I] = 10 mM and [X] = 40 mM (f), [I] = 30 mM and [X] = 40 mM (g).

The double reciprocal Lineweaver-Burk plots[17,18] for urea catalysis by jack bean urease in different fixed concentrations of I and X, at pH = 7.0 and T = 27°C are shown in Figure 1. The value of V_{max} is unchanged by the compounds I and X, but the apparent Michaelis constant (K'_m) value is increased, which confirms the synergistic competitive inhibition by I and X of urease. The value of V_{max} given is 105 μmole min^{-1}. The values of K'_m at any fixed concentration of I and X are obtained from Figure 1 and are tabulated in Table I.

Equation (5) can be rearranged to:

$$\frac{1}{K_I} = \frac{(K'_m / K_m) - 1}{[I]} - ([X] / [I])\frac{1}{K_X} \qquad (6)$$

The plot of $1/K_I$ versus $1/K_X$ at any fixed concentration of I and X, from the known values of K'_m and K_m and in the reasonable range of values for K_I and K_X is linear (Figure 2). Each line contains a set of points with $1/K_I$ and $1/K_X$ coordinates, but there is only one point on each line which is consistent with real values of K_I and K_X. The lines intersect at a focus point that gives the values of K_I and K_X which are 26 and 28 mM, respectively.

TABLE I The values for the Michaelis constants at different concentrations of I and X obtained from Lineweaver-Burk plots.

[I] mM	0	5	10	20	30	10	30
[X] mM	0	5	5	5	10	40	40
K'_m mM	2.69	3.69	4.18	5.27	6.80	7.54	9.67

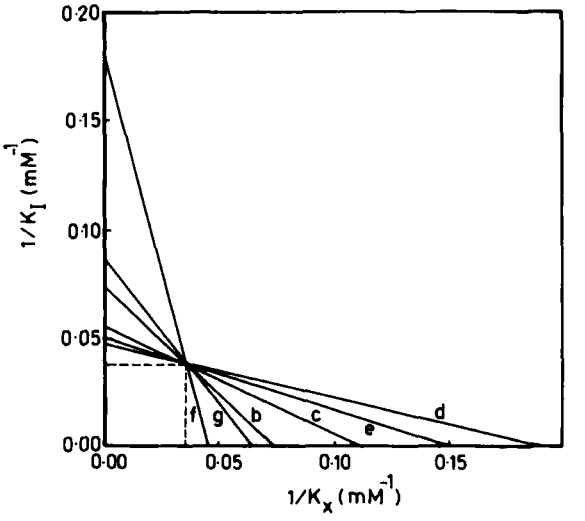

FIGURE 2 The plots of $1/K_I$ versus $1/K_X$ for a reasonable range of values for the inhibition constants using different observed values of the apparent Michaelis constant: K'_m = 3.69 mM (b), K'_m = 4.18 mM (c), K'_m = 5.27 mM (d), K'_m = 6.80 mM (e), K'_m = 7.54 mM (f), K'_m = 9.67 mM (g). The calculation uses equation (6) with K_m = 2.69 mM.

It is important to note that the results obtained from this method were confirmed by secondary Lineweaver-Burk plots. The results obtained from the two methods were identical. Therefore, our method has simplicity and is less time-consuming than other methods since the number of assays is diminished.

Acknowledgements

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