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ENTHALPIMETRIC AND COLORIMETRIC DETERMINATIONS OF THE INHIBITION CONSTANT FOR THE INHIBITION OF UREASE BY BORIC ACID

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Abstract

Continuous enthalpimetric and fixed-time-point colorimetric analytical studies of jack bean urease-catalyzed hydrolysis of urea uninhibited and inhibited by boric acid were performed. The effect of boric acid on urease activity was studied in 100 mM phosphate buffer of pH 7 (H_3PO_4+NaOH, 2 mM EDTA) at 25°C. The inhibition constant of boric acid was obtained with use of differential and integration methods. It was found that boric acid is a simple competitive inhibitor of urease. The methods produced comparable values of the inhibition constant K_i: the former 0.19 mM and the latter 0.18 mM.

Keywords: boric acid, enzymatic enthalpimetry, inhibition, kinetic integration methods, urease

Introduction

Enthalpimetry has been successfully applied in kinetic studies of enzymatic reactions [1]. The technique permits continuous recording of the reaction progress, and it is therefore especially useful in studies of reactions which are difficult or impossible to follow continuously by means of other analytical methods. One of them is the urease-catalyzed hydrolysis of urea. Enthalpimetric determinations of kinetic constants of urease [2–5] and of inhibition constants of a number of urease inhibitors have been reported [6–8]. In this study the results of monitoring of the process by enthalpimetry and by fixed-time-point colorimetric analysis are compared, both applied here for kinetic methods based on the integrated Michaelis-Menten equation. The results are compared with those obtained from analytical measurements by methods based on the differential Michaelis-Menten equation.

Commonly applied differential methods of determination of inhibition constants K_i based on initial reaction rate measurements, monitor the early short phase of the reaction, whereas integration methods based on reaction progress curves monitor the whole duration of the reaction up to the exhaustion of the sub-

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strate [9]. Application of both groups of methods provides verification of the determinations, i.e. for undisturbed inhibition the same value of K_i should be obtained.

Integration methods are more economic than differential methods, as they require recording of the progress curves of two reactions carried out in the absence and presence of an inhibitor at a chosen substrate concentration, whereas differential methods require measurements of initial rates of reactions carried out for a series of inhibitor concentrations at a series of substrate concentrations. Reaction progress curves can be obtained either by analytical determination of substrate or product concentrations at various time intervals throughout the time course of a reaction, or by continuous recording by e.g. enthalpimetry. The latter considerably simplifies the experiment.

The kinetics of inhibition of urease has been extensively studied [10]. Inhibitors such as hydroxamic acids [11–13], phosphoroamide compounds [12], boric and boronic acids [12, 14], are considered as potential therapeutic agents against bacterial urease-induced human pathogenic states such as urinary stone formation and gastric ulceration, and as agents controlling the level of urea and ammonia in soil after use of urea fertilizers [10]. Two bacterial ureases: *Proteus mirabilis* and *Klebsiella aerogenes* were studied by Breitenbach and Hausinger [14] and by Todd and Hausinger [12] for their inhibition by boric acid. Boric acid was found to be a competitive inhibitor of both of them with the inhibition constant K_i equal to 0.1 mM for the former and 0.33 mM for the latter.

In this study the kinetics of inhibition of jack bean urease by boric acid was investigated by colorimetric analytical and enthalpimetric techniques with use of differential and integration methods. The reaction was carried out in phosphate buffer pH 7 (2 mM EDTA) prepared by neutralization of orthophosphoric acid with NaOH [3, 15]. The inhibitory strength of boric acid was compared with those of other jack bean urease inhibitors.

Symbols and basic equations

S_o, S	substrate (urea) concentration, initial and remaining after time t, mM.
P_o, P	product concentration, initial and formed in time t, mM, $P=[NH_3]/2$,
K_M	Michaelis constant, mM,
v	reaction rate, mM s^{-1}, $v=dP/dt$,
I	inhibitor (H_3BO_3) concentration, mM.
K_i	inhibition constant, mM,
ε	fractional conversion,
ΔT, ΔT_{max}	corrected temperature increments, transient and final, °C,
m, n	coefficients of straight lines, slope and intercept.

The following relationships are valid: $P+S=S_o$, $S=S_o\,(1-\varepsilon)$, and $\varepsilon=\Delta T/\Delta T_{max}$. The kinetics of urease-catalyzed hydrolysis of urea, except for high urea and ammonia concentrations, is well described by the differential Michaelis-Menten equation [16]:

$$v = -\frac{dS}{dt} = \frac{v_{max} S}{K_M + S} \tag{1}$$

If boric acid is a simple competitive inhibitor of urease, the reactions taking place in the system are described by the following scheme:

$$\begin{array}{l} E + S \Leftrightarrow ES \rightarrow EP \\ + \\ I \\ \Updownarrow \\ EI \end{array} \tag{2}$$

Eq. (1) changes into:

$$v = \frac{v_{max} S}{K_i\left(1 + \frac{I}{K_i}\right) + S} \tag{3}$$

Equation (3) can be integrated in two different ways: one leads to Eq. (4), the other to Eq. (5) [9]:

$$\frac{P}{v_{max}} + \frac{K_M}{v_{max}}\left(1 + \frac{I}{K_i}\right)\ln\frac{S_o}{S_o - P} = t \tag{4}$$

$$K_M\left(1 + \frac{I}{K_i}\right)(S - S_o) + \frac{1}{2}(S^2 - S_o^2) = -v_{max}\int_o^t S\,dt \tag{5}$$

All the equations used in this study are derived from differential equation (3) or its integrated forms (4) and (5). The former is the basis of differential methods, and the latter of integration methods of K_i determination.

Experimental

Jack bean urease, Sigma type III, with specific activity 33 units/mg protein, was used. One unit of activity is defined as the amount of enzyme that liberates 1.0 μmol NH_3 from urea per minute at pH 7 and 25°C. Urea, boric acid and other chemicals (analar grade) were from POCh, Gliwice, Poland.

Urease-catalyzed hydrolysis of urea: $(NH_2)_2CO+H_2O\rightarrow 2NH_3+CO_2$, in the absence and presence of boric acid was carried out in 100 mM phosphate buffer

pH 7 containing 2 mM EDTA, at 25°C. The buffer was prepared from orthophosphoric acid solution titrated potentiometrically with NaOH solution to pH 7. 100 mM was the lowest buffer concentration necessary to keep the pH of the reaction mixture unchanged throughout the whole period of reaction. The reaction was initiated by addition of urease to urea- and urea-boric acid solutions, respectively.

The reaction was monitored by two techniques: analytical (colorimetric) and enthalpimetric. In the former samples were taken from the reaction mixture at intervals for ammonia determination by the colorimetric phenol-hypochlorite method [17]. In the latter the reaction was carried out in the isoperibol set and the rate of change in temperature was registered with an accuracy of 0.001°C at 2 s intervals. The detailed description of the calorimetric equipment was presented previously [7].

Differential methods (measurements of initial reaction rates)

The reaction was carried out in the presence of the following inhibitor concentrations: 0, 0.25, 0.5, 1 and 2 mM H_3BO_3. For each inhibitor concentration the initial rate of the reaction was measured in solutions of urea concentrations between 2 and 50 mM. The volume of the reaction mixture was 25 cm^3, and the concentration of urease 0.025 mg cm^{-3}. The initial reaction rates were determined by the colorimetric analytical method.

Integration methods (recording of reaction progress curves)

The progress curves of the reaction in the absence and presence of the inhibitor (0.25 mM H_3BO_3) were recorded in 10 mM solutions of urea. The concentration of urease in both solutions was 0.1 mg cm^{-3}. The curves were recorded both by the colorimetric analytical (time intervals: 0.5 min) and enthalpimetric method.

Results

K_i from differential methods

To obtain K_i of boric acid from the measured initial rates of urease-catalyzed urea hydrolysis carried out in the absence and presence of boric acid of different concentrations, the Lineweaver-Burk and Hanes linear transformations of Eq. (3) were used [9].

(i) The Lineweaver-Burk transformation:

The rate equation (3) for competitive inhibition in the Lineweaver-Burk form is:

$$\frac{1}{v} = \frac{K_M}{v_{max}}\left(1 + \frac{I}{K_i}\right)\frac{1}{S} + \frac{1}{v_{max}} \qquad (6)$$

The straight lines $1/v$ *vs.* $1/S$ for different inhibitor concentrations intersect at the point $(0, 1/v_{max})$. The slopes of the above lines in the presence of an inhibitor are given by:

$$\text{slope} = \frac{K_M}{v_{max} K_i} I + \frac{K_M}{v_{max}} \tag{7}$$

A plot of the slope (7) *vs.* inhibitor concentration allows to calculate the value of K_i.

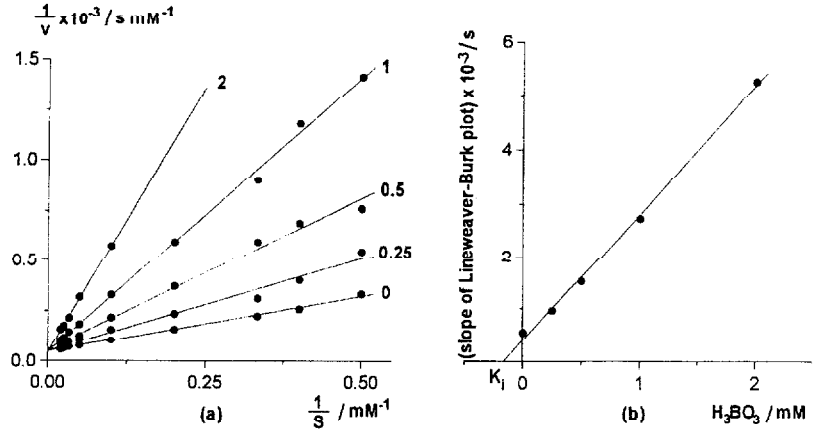

Fig. 1 (a) Lineweaver-Burk plot ($1/v$ *vs.* $1/S$) for the urease-catalyzed hydrolysis of urea in the presence of different concentrations of H_3BO_3. Numbers denote the concentration of H_3BO_3, mM (b). Plot of the slope of Lineweaver-Burk plots *vs.* inhibitor concentration

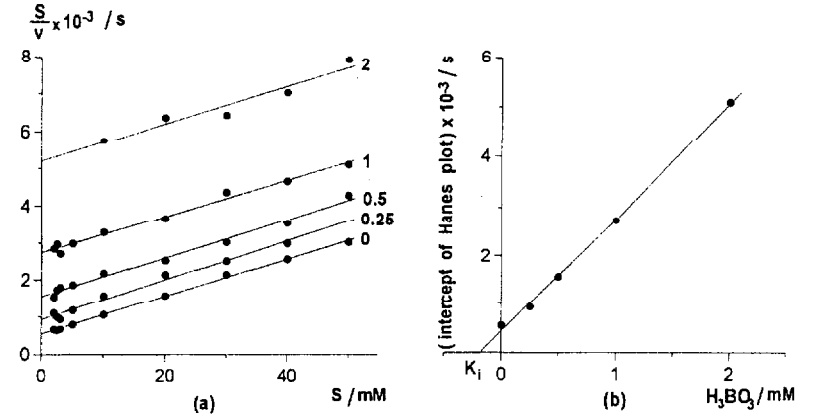

Fig. 2 (a) Hanes plot (S/v *vs.* S) for the urease-catalyzed hydrolysis of urea in the presence of different concentrations of H_3BO_3. Numbers denote the concentration of H_3BO_3, mM (b). Plot of the intercept of Hanes plots *vs.* inhibitor concentration

The results thus obtained for the studied system are presented in Fig. 1a and 1b. The value of K_i is determined as 0.18 mM.

(ii) The Hanes transformation:

The rate equation (3) used in the Hanes transformation is:

$$\frac{S}{v} = \frac{1}{v_{max}} S + \frac{K_M}{v_{max}} \left(1 + \frac{I}{K_i}\right) \tag{8}$$

The lines S/v *vs.* S for different inhibitor concentrations are parallel. Their intercepts are given by:

$$\text{intercept} = \frac{K_M}{v_{max} K_i} I + \frac{K_M}{v_{max}} \tag{9}$$

A plot of the intercept (9) *vs.* inhibitor concentration provides the value of K_i. The obtained results are presented in Fig. 2a and 2b. The value of K_i is determined as 0.20 mM.

The location of the linear plots in Fig. 1a intersecting at one point $(0, 1/v_{max})$ and parallel in Fig. 2a proves that the inhibition of jack bean urease by boric acid is of a simple competitive type.

K_i from integration methods

The reaction progress curves recorded colorimetrically and enthalpimetrically for the urease-catalyzed hydrolysis of urea (10 mM), uninhibited and inhibited by 0.25 mM boric acid, are presented in Fig. 3a and 3b, respectively. The

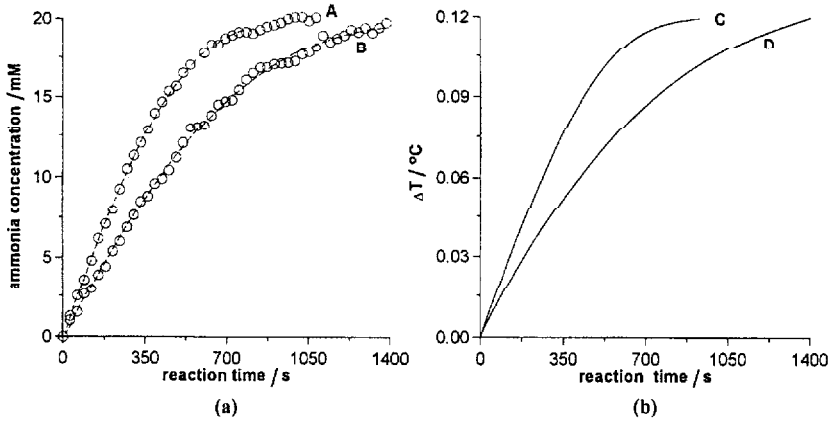

Fig. 3 Reaction progress curves recorded for the urease-catalyzed hydrolysis of urea by means of colorimetric analytical (a) and enthalpimetric technique (b); curves A and C for the uninhibited reaction; curves B and D for the reaction inhibited with 0.25 mM H_3BO_3. Curves A and B approximated by polynomials of the third degree (dotted lines)

analytical progress curves A and B, ammonia concentration *vs.* time, were approximated by polynomials of the third degree in the range $0<\varepsilon<0.94$ (dotted lines). The enthalpimetric progress curves C and D, temperature increment *vs.* time, were converted into ammonia concentration in the following way: $[NH_3]=2[urea]_{t=0}\Delta T/\Delta T_{max}$, with $\Delta T_{max}=0.1200°C$, and described by polynomials

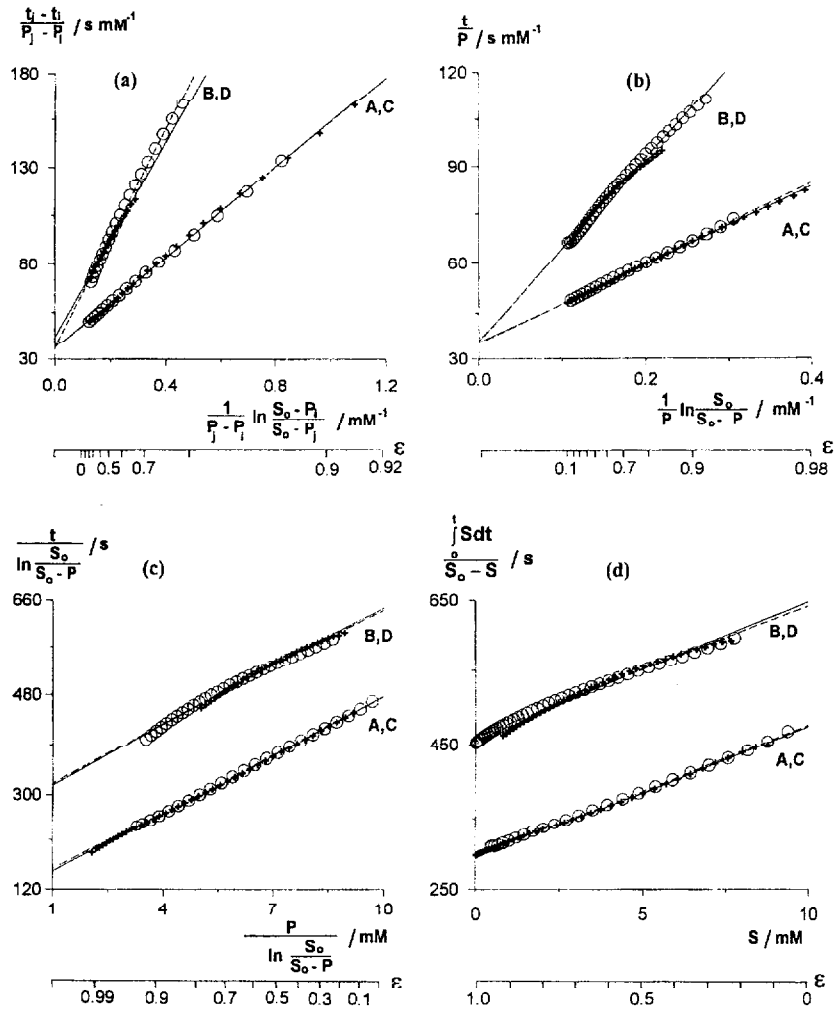

Fig. 4 Plots of the progress curves of Fig. 3 obtained by the integration methods of (a) Yun-Suelter, (b) Jennings-Niemann (I), (c) Jennings-Niemann (II), and (d) Booman-Niemann. A, C – uninhibited reaction; B, D – inhibited reaction. A, B – colorimetric analytical technique (o), dotted lines; C, D – enthalpimetric technique (+), solid lines. The added ε-axes show the sections of the progress curves from which the linear plots were obtained

of the third degree in the range $0<\varepsilon<0.96$. There is good agreement between curves A and C for the uninhibited reaction, and between B and D for the inhibited reaction, which will be further reflected by the corresponding linear plots (Fig. 4) and in the values obtained for K_i (Table 1).

To obtain K_i of boric acid from the recorded progress curves, four linearization procedures based on the integrated rate equations (4) and (5) were applied.

(i) The Yun-Suelter procedure [18]:

The procedure employs the integrated rate equation (4) in the form:

$$\frac{t_j - t_i}{P_j - P_i} = \frac{K_M}{v_{max}}\left(1 + \frac{I}{K_i}\right)\frac{1}{P_i - P_i}\ln\frac{S_o - P_i}{S_o - P_i} + \frac{1}{v_{max}} \tag{10}$$

where P_j and P_i are product concentrations corresponding to time t_j and t_i of the reaction, respectively. A plot of $\frac{t_j - t_i}{P_j - P_i}$ *vs.* $\frac{1}{P_j - P_i}\ln\frac{S_o - P_i}{S_o - P_j}$ is linear with a slope $m = \frac{K_M}{v_{max}}\left(1 + \frac{I}{K_i}\right)$. The linear plots of the polynomial progress curves A, B, C and D are presented in Fig. 4a. The values of K_i were calculated from the following relationships for colorimetric analytical and enthalpimetric plots, respectively:

$$\left(\frac{K_i}{I}\right)_{anal} = \frac{m_A}{m_B - m_A}, \quad K_i=0.17 \text{ mM}$$

$$\left(\frac{K_i}{I}\right)_{enthal} = \frac{m_C}{m_D - m_C}, \quad K_i=0.17 \text{ mM}$$

(ii) The Jennings-Niemann procedure (I) [19]:

The following form the integrated rate equation (4) is used:

$$\frac{t}{P} = \frac{K_M}{v_{max}}\left(1 + \frac{I}{K_i}\right)\frac{1}{P}\ln\frac{S_o}{S_o - P} + \frac{1}{v_{max}} \tag{11}$$

The plot of $\frac{t}{P}$ *vs.* $\frac{1}{P}\ln\frac{S_o}{S_o - P}$ is linear with a slope $m = \frac{K_M}{v_{max}}\left(1 + \frac{I}{K_i}\right)$. The linear plots of the polynomial progress curves A, B, C and D are presented in Fig. 4b. The values of K_i calculated from the colorimetric analytical and enthalpimetric plots are, respectively:

$$\left(\frac{K_i}{I}\right)_{anal} = \frac{m_A}{m_B - m_A}, \quad K_i=0.19 \text{ mM}$$

$$\left(\frac{K_i}{I}\right)_{enthal} = \frac{m_C}{m_D - m_C}, \quad K_i = 0.18 \text{ mM}$$

(iii) The Jennings-Niemann procedure (II) [19]:
For the following form of Eq. (4):

$$\frac{t}{\ln\frac{S_o}{S_o - P}} = \frac{1}{v_{max}} \frac{P}{\ln\frac{S_o}{S_o - P}} + \frac{K_M}{v_{max}}\left(1 + \frac{I}{K_i}\right) \tag{12}$$

the plot of $\frac{t}{\ln\frac{S_o}{S_o - P}}$ *vs.* $\frac{P}{\ln\frac{S_o}{S_o - P}}$ is linear with an intercept $n = \frac{K_M}{v_{max}}\left(1 + \frac{I}{K_i}\right)$. Figure 4c shows the linear plots of the polynomial curves A, B, C and D from which the values of K_i were calculated:

$$\left(\frac{K_i}{I}\right)_{anal} = \frac{n_A}{n_B - n_A}, \quad K_i = 0.19 \text{ mM}$$

$$\left(\frac{K_i}{I}\right)_{enthal} = \frac{n_C}{n_D - n_C}, \quad K_i = 0.18 \text{ mM}$$

(iv) The Booman-Niemann procedure [20]:
The procedure employs Eq. (5) in the form:

$$\frac{\int_o^t S\mathrm{d}t}{S_o - S} = \frac{1}{2v_{max}} S + \frac{2K_M\left(1 + \frac{I}{K_i}\right) + S_o}{2v_{max}} \tag{13}$$

The integrals $\int_o^t S\mathrm{d}t$ were calculated by integrating the approximating polynomials. The plot of $\frac{\int_o^t S\mathrm{d}t}{S_o - S}$ *vs.* S is linear with a slope $m = \frac{1}{2v_{max}}$ and intercept $n = \frac{2K_M\left(1 + \frac{I}{K_i}\right) + S_o}{2v_{max}}$. Figure 4d shows the linear plots of the polynomial curves A, B, C and D. The values of K_i were calculated:

$$\left(\frac{K_i}{I}\right)_{anal} = \frac{n_A - m_A S_o}{n_B - n_A}, \quad K_i = 0.18 \text{ mM}$$

$$\left(\frac{K_i}{I}\right)_{enthal} = \frac{n_C - m_C S_o}{n_D - n_C}, \qquad K_i = 0.18 \text{ mM}$$

The linearization ranges (expressed by ε) applied in the above linearization procedures are given in respective figures. The pairs of plots representing the two studied reactions have either the same intercept (Fig. 4a and 4b) or the same slope (Fig. 4c and 4d) providing evidence that boric acid is a simple competitive inhibitor of jack bean urease.

Table 1 Inhibition constant K_i, mM, of boric acid for the urease-catalyzed hydrolysis of urea determined by the applied differential and integration methods

Method	K_i/mM	
	Monitoring technique	
	colorimetric	enthalpimetric
Differential:		
Lineweaver-Burk	0.18	–
Hanes	0.20	–
mean value	0.19±0.01	
Integration:		
Yun-Suelter	0.17	0.17
Jennings-Niemann (I)	0.19	0.18
Jennings-Niemann(II)	0.19	0.18
Booman-Niemann	0.18	0.18
mean value	0.18±0.01	

The values obtained for the inhibition constant K_i of boric acid for urease-catalyzed hydrolysis of urea are listed in Table 1.

Discussion

Both groups of the applied methods: differential and integration showed that boric acid is a simple competitive inhibitor of jack bean urease in phosphate buffer of pH 7 (100 mM, H_3PO_4+NaOH, 2 mM EDTA), 25°C, with the inhibition constant K_i equal to 0.19 and 0.18 mM as obtained by differential and integration methods, respectively. The above findings are consistent with those concerning bacterial ureases [12, 14].

Both groups of the applied methods produced comparable results. The two differential methods applied, those of Lineweaver-Burk and Hanes are supplementary to each other, as the former gives results dependent on initial rates at low

substrate concentrations, and the latter on those at high substrate concentrations. Integration methods applied to the progress curves recorded by means of colorimetric analytical method, by taking points at time intervals throughout the time course of the reaction, and in an enthalpimetric continuous way, gave consistent results. The study shows that integration methods are equally reliable in the investigation of the kinetics of enzyme inhibition as conventionally used differential methods, thus providing a fast and economic tool in enzyme analysis. Of the two techniques used, enthalpimetric, though more difficult to interpret, permits advantageous continuous recording.

Table 2 Selected competitive inhibitors of jack bean urease

Inhibitor	Buffer	pH	K_i/mM	Ref.
Competitive:				
2-mercaptoethanol	phosphate	7.0	0.95±0.01	[21]
	HEPES, phosphate	7.1	0.72±0.26	[22]
	N-ethylmorpholine-HCl	7.1	0.95±0.05	[22]
Boric acid	phosphate	7.0	0.19±0.01	this paper
Na-Phosphate	phosphate	7.0	31	[23]
Competitive slow-binding:				
Acetohydroxamic acid	phosphate	7.0	0.026	[8]
Sodium fluoride	phosphate	7.0	0.029	[24]

The K_i value is a measure of inhibitory strength of an inhibitor. The lower the value of K_i, the greater the degree of inhibition at any given concentrations of S and I. In Table 2 boric acid is compared with other competitive inhibitors of jack bean urease: 2-mercaptoethanol and phosphate, and with competitive slow-binding inhibitors: acetohydroxamic acid and sodium fluoride. The K_i value of boric acid classifies it as an inhibitor of moderate inhibitory strength.

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