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Thermal Analysis

Inhibition of jack bean urease by thiols. Calorimetric studies

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Abstract

The influence of three thiol compounds: β-mercaptoethylamine thioglycolic acid and thioacetic acid on the urease-catalyzed hydrolysis of urea was studied. The reaction was carried out in phosphate buffer pH 7.0 at 25°C. The progress of the reaction was observed by means of a calorimetric method using an isoperibol calorimeter. The total reaction progress curves, ΔT vs. t, were interpreted with the help of the integration Jennings–Niemann method and the inhibition constants were calculated.

Keywords: Urease; Inhibition; Thiols; Calorimetry

1. Introduction

Most enzymatic reactions are exothermic and associated with rather high enthalpy changes which make calorimetry a very effective experimental technique, insensitive to the optical or electrochemical properties of sample. Urease-catalyzed hydrolysis of urea: $CO(NH_2)_2+H_2O\rightarrow 2NH_3+CO_2$ has a significant positive thermal effect which strongly depends on the experimental conditions. Buffer system and pH value have great influence on the values of molar enthalpy of enzymatic hydrolysis of urea [1–3]. The highest enthalpy change was observed in phosphate buffer at pH 7.5 and was equal to −61.3 kJ/mol [1].

Urease is widely distributed in a variety of bacteria, fungi and plants. Enzymes obtained from different sources differ in structure, number and type of subunits, molecular weight and amino acid sequence [4]. The best-characterised urease is the enzyme purified from the plant *Canivalia ensiformis*, jack bean. Jack bean urease was the first enzyme to be crystallised and also the first shown to contain nickel. Jack bean urease is a homohexameric enzyme with 2 mol of nickel per mole of subunit. Zerner et al. [5] have proposed a model of the active site of urease. The resting enzyme at neutral pH contains a water molecule coordinated to one of the nickel ions and a hydroxide ion coordinated to the other. Three amino acid residues: a sulfhydryl group a carboxyl group and an undefined base are also important for the catalytic activity of urease. In the course of the catalysis urea forms a tetrahedral intermediate which bridges two nickel ions. Spectroscopic studies suggested that the distance between two nickel ions is ca 3.5 Å and each metal ion is approximately

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pentacoordinate, with two imidazoles and additional N or O atoms serving as ligands [6]. Karplus et al. [7,8] have accommodated Zerner's model for *Klesiella aerogenes* urease.

Urease inhibitors inactivate enzyme in a variety of ways. Hydroxamic acids and phosphoroamide compounds create a tetrahedral intermediate with a structural similarity to the tetrahedral intermediate postulated to occur during urea hydrolysis [9–11]. Heavy metal ions react with the active site sulfhydryl group. The reaction is analogous to the formation of metal sulfide [12]. Boric and boronic acids are suggested to form a complex with nickel ion(s) [13]. Several thiol compounds were shown to be competitive inhibitors of *Klesiella aerogenes* urease. Thiols are not potent inhibitors however the presence of other charged groups has a significant effect on the inhibition constant [9]. For jack bean urease among thiols only β-mercaptoethanol was examined as an inhibitor [14].

The phenol-hypochlorite colorimetric method is commonly used for study of inhibition of urease-catalyzed hydrolysis of urea. This method cannot be applied for some compounds, e.g. those containing –SH group because of an interference with the phenol-hypochlorite assay [15]. Todd et al. [9] used an ammonia-selective electrode for study of thiol compounds. The pH-stat and spectrophotometric methods have been applied for the determination of the inhibition constant of β-mercaptoethanol by Dixon et al. [14]. Zaborska [16] made the separate calibration curves for ammonium ions using a phenol-hypochlorite method for study of thioglycolic acid.

In the previous report [17], the calorimetric technique combined with four integral calculation methods has been successfully used for the determination of the inhibition constant of β-mercaptoethanol. In this study, the kinetics of inhibition of jack bean urease by β-mercaptoethylamine, thioglycolic acid and thioacetic acid was investigated by the calorimetric technique with use of the integration Jennings–Niemann method. The obtained values of the inhibition constants K_i of β-mercaptoethylamine and thioglycolic acid are compared with the values determined for *Klebsiella aerogenes* urease. The inhibitory strength of thiols is interpreted in terms of a model of urease active site and thiol molecules structure.

2. Symbols and basic equations

S_0, S — substrate (urea) concentrations; initial and remaining after time t (mmol dm^{-3}),
P — product concentration (mmol dm^{-3}), P=[NH_3]/2,
K_M — Michaelis constant (mmol dm^{-3}),
v — reaction rate (mmol dm^{-3} s^{-1}), v=dP/dt,
K_i — inhibition constant (mmol dm^{-3}),
ΔT, ΔT_{max} — corrected heat increments; transient and final (°C),
ε — fraction conversion,
m_0, m_I — slopes of straight lines, in the absence and presence of inhibitor at [I] concentration.

The following relationships are in place:

$$P + S = S_0, \quad S = S_0(1 - \varepsilon) \text{ and } \varepsilon = \Delta T/\Delta T_{max} \quad (1)$$

An urease-catalyzed hydrolysis of urea ($E+S \Leftrightarrow ES \rightarrow E+P$) is well described by the hyperbolic Michaelis–Menten equation Eq. (2):

$$v = -\frac{dS}{dt} = \frac{v_{max} S}{K_M + S} \quad (2)$$

After integration Eq. (2) becomes:

$$t = \frac{K_M}{v_{max}} \ln\left(\frac{S_0}{S_0 - P}\right) + \frac{P}{v_{max}} \quad (3)$$

Eq. (3) can be linearized in many ways [18–21]. The Jennings–Niemann procedure [18] employs an integrated rate equation Eq. (3) in the form:

$$\frac{t}{P} = \frac{K_M}{v_{max}} \frac{1}{P} \ln\left(\frac{S_0}{S_0 - P}\right) + \frac{1}{v_{max}} \quad (4)$$

Transformation of a progress curve into coordinate system t/P vs. $1/P \ln(S_0/(S_0-P))$ gives a linear replot with a slope K_M/v_{max} and intercept $1/v_{max}$.

In presence of a simple competitive inhibitor the reaction taking place in the system is described by the scheme:

$$E + S \Leftrightarrow ES \rightarrow E + P$$
$$E + I \Leftrightarrow EI$$

Eq. (4) for competitively inhibited reaction changes into Eq. (5):

$$\frac{t}{P} = \frac{K_M}{v_{max}}\left(1 + \frac{I}{K_i}\right) \frac{1}{P} \ln\left(\frac{S_0}{S_0 - P}\right) + \frac{1}{v_{max}} \quad (5)$$

Application of the Jennings–Niemann linearization procedure transforms reaction progress curves monitoring the reactions in the absence and presence of inhibitor into straight lines which intersect at one point corresponding to $1/v_{max}$ and have different slopes: equal to $m_0=K_M/v_{max}$ and $m_I=K_M/v_{max}$ $(1+(I/K_i))$, respectively. The inhibition constant can be calculated with the use of the following proportion:

$$\frac{K_i}{I}=\frac{m_0}{m_I-m_0} \quad (6)$$

2.1. Materials

The jack bean urease, Sigma type III of specific activity 33 units/mg protein was used. One unit is the amount of enzyme that liberates 1.0 μmol of NH_3 from urea per minute at pH 7 and 25°C. β-Mercaptoethylamine, thioacetic acid, urea (Molecular Biology Reagent) were purchased from Sigma. Thioglycolic acid was from Aldrich. Other chemicals were obtained from POCh, Gliwice, Poland.

All used reagents were of analytical grade (p. A.).

2.2. Enzymatic reaction

The hydrolysis of urea catalyzed by jack bean urease was studied in phosphate buffer pH 7.0 (100 mmol dm^{-3}, 2 mmol dm^{-3} EDTA) at 25°C. The initial concentration of urea was 10 mmol dm^{-3}. The studied concentrations of thiols: β-mercaptoethylamine, 0.05 and 0.1 mmol dm^{-3}; thioglycolic acid, 2 and 3 mmol dm^{-3}; thioacetic acid, 1 and 2 mmol dm^{-3}.

The reaction was initiated by addition of 0.5 cm^3 of urease solution (20 mg cm^{-3}) into 100 cm^3 of assay mixture.

2.3. Experimental techniques

The progress of urea hydrolysis in the absence and presence of inhibitors was observed in the isoperibol calorimetric set. Before the reaction in the calorimeter, the investigated systems were thermostatted to a constant temperature of 25.000 ± 0.001°C. The observed differential increments ΔT were corrected for a heat exchange.

The details of the calorimetric measurements and their evaluation have been communicated in an earlier publication [22].

3. Results

The reaction progress curves in the absence and presence of inhibitor were recorded and corrected for the heat exchange. The experimental data were approximated with polynomials of the fourth degree.

3.1. Reaction without inhibitor

The total amount of urea in the reaction mixture was hydrolyzed after ca. 15 min and the system reached

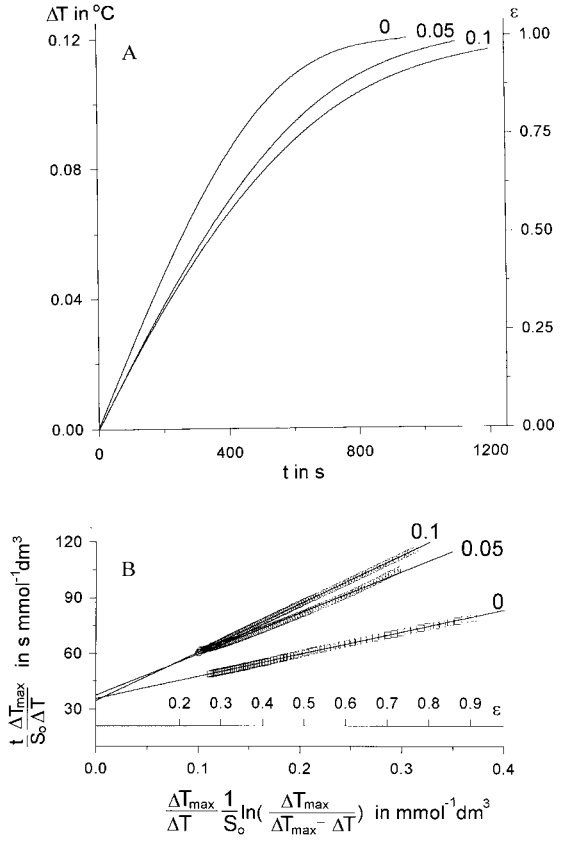


Fig. 1. (A) Reaction progress curves of urease-catalyzed hydrolysis of urea recorded by the calorimetric technique; curve 0 for the uninhibited reaction; curves 0.05 and 0.1 for the reaction inhibited with 0.05 and 0.1 mmol dm^{-3} β-mercaptoethylamine. Curves approximated with polynomials of the fourth degree. (B) Replots of the progress curves obtained by the integration Jennings–Niemann method. The added ε-axes show the sections of the progress curves expressed in terms of ε, from which the linear replots were obtained.

the maximum increment of temperature equal to ΔT_{max}=0.1200°C. The uninhibited reaction progress curve was a reference curve for all reaction progress curves in the presence of inhibitor and is presented in Fig. 1A, Fig. 2A and Fig. 3A as a function with a number 0.

3.2. Reaction with inhibitor

Two different concentrations of each thiol were studied. The registered progress curves for β-mercaptoethylamine, thioacetic acid and thioglycolic acid, ΔT vs. t, are presented in Fig. 1A, Fig. 2A and Fig. 3A, respectively. The axis on the right-hand side expresses the fraction conversion ε.

3.3. Calculation of the inhibition constants K_i

Introduction of calorimetric parameters Eq. (1) to Eq. (5) gives the calorimetric form of the Jennings–Niemann integrated equation Eq. (7):

$$\frac{t}{S_0}\frac{\Delta T_{max}}{\Delta T} = \frac{K_M}{v_{max}}\left(1+\frac{I}{K_i}\right)\frac{\Delta T_{max}}{\Delta T}\frac{1}{S_0} \times \ln\left(\frac{\Delta T_{max}}{\Delta T_{max}-\Delta T}\right)+\frac{1}{v_{max}} \quad (7)$$

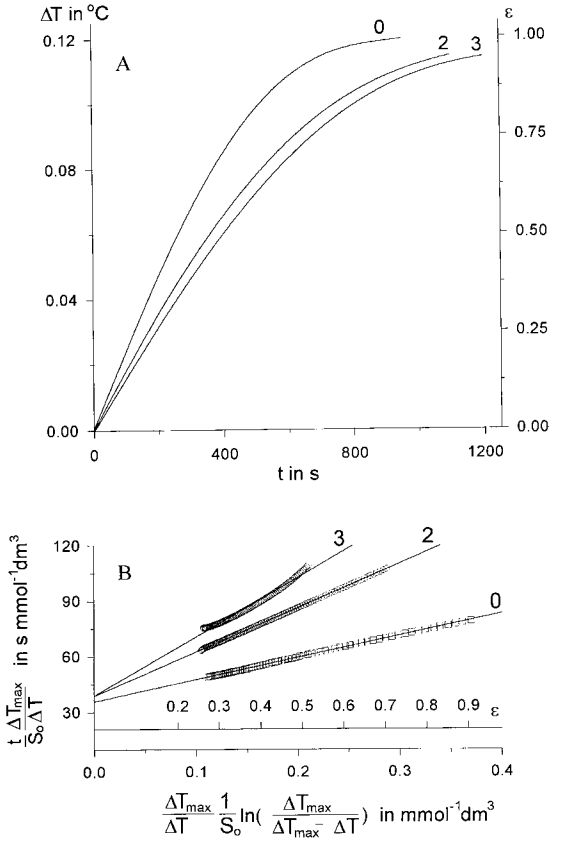


Fig. 2. (A) Reaction progress curves of urease-catalyzed hydrolysis of urea recorded by the calorimetric technique; curve 0 for the uninhibited reaction; curves 2 and 3 for the reaction inhibited with 2 and 3 mmol dm^{-3} thioglycolic acid. Curves approximated with polynomials of the fourth degree. (B) Replots of the progress curves obtained by the integration Jennings–Niemann method. The added ε-axes show the sections of the progress curves expressed in terms of ε, from which the linear replots were obtained.

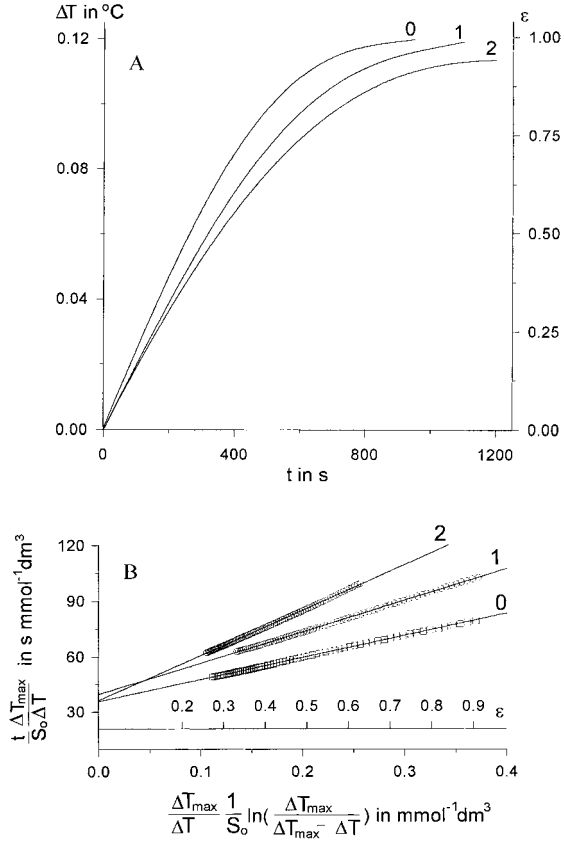


Fig. 3. (A) Reaction progress curves of urease-catalyzed hydrolysis of urea recorded by the calorimetric technique; curve 0 for the uninhibited reaction; curves 1 and 2 for the reaction inhibited with 1 and 2 mmol dm^{-3} thioacetic acid. Curves approximated with polynomials of the fourth degree. (B) Replots of the progress curves obtained by the integration Jennings–Niemann method. The added ε-axes show the sections of the progress curves expressed in terms of ε, from which the linear replots were obtained.

Table 1
Inhibition constants of urea hydrolysis catalyzed by jack bean urease inhibited by thiols obtained by the integration Jennings–Niemann method, 100 mmol dm^{-3} phosphate buffer pH 7.0

Thiol	Concentration (mmol dm^{-3})	Total inhibitor K_i (mmol dm^{-3})	Mean value K_i (mmol dm^{-3})
β-Mercaptoethylamine	0.05	0.059	0.067 ± 0.011
	0.1	0.075	
Thioglycolic acid	2	2.2	2.1±0.21
	3	1.9	
Thioacetic acid	1	3.0	2.5±0.78
	2	1.9	

where S_0 and ΔT_{max} are fixed for all studied systems: S_0=10 mmol dm^{-3}, ΔT_{max}=0.1200°C. The transformation of reaction progress curves, ΔT vs. t, into linearization coordinate system

$$\frac{t}{S_0}\frac{\Delta T_{max}}{\Delta T} \quad \text{vs.} \quad \frac{\Delta T_{max}}{\Delta T}\frac{1}{S_0}\ln\left(\frac{\Delta T_{max}}{\Delta T_{max}-\Delta T}\right)$$

results in linear replots.

The integration Jennings–Niemann procedure is not effective for the linearization of the total reaction progress curve, especially, the beginning and final stage of the reaction are less reliable. The restrictions of the method have been reported previously [23].

The linear replots of the progress curves are presented in Fig. 1B, Fig. 2B and Fig. 3B. The linearization range (expressed with ε) applied in the above linearization procedure is given in respective figures. The two values of the inhibition constants for lower and higher concentration of each thiol were obtained from the values of parameters of the linear replots and the mean values were calculated. The results are presented in Table 1.

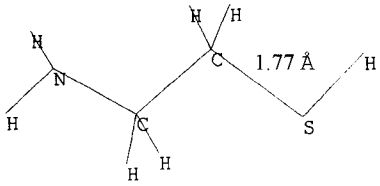
4. Discussion

The kinetics of the inhibition of jack bean urease by three thiols: β-mercaptoethylamine; thioglycolic acid; and thioacetic acid were interpreted with use of the integrated Michaelis–Menten equation: Jennings–Niemann procedure. Obtained linear replots of the total progress curves, in the absence and presence of inhibitor, intersect at one point equal to $1/v_{max}$ and have different slopes (Figs. 1–3). The foregoing proves that β-mercaptoethylamine, thioglycolic acid and thioacetic acid are competitive inhibitors of jack bean urease. Obtained inhibition constants (including β-mercaptoethanol [17]) show that the inhibitory strength grows at the following direction:

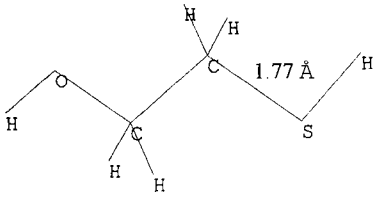
Table 2
Inhibition constants of plant (jack bean, *Canivalia ensiformis*) and bacterial urease (*Klebsiella aerogenes*) by thiols

Thiol	K_i (mmol dm^{-3})		
	Jack bean urease		Bacterial urease
	Calorimertric method	Other method	Ammonia-selective electrode method
β-Mercaptoethylamine	0.067 [This paper]		0.010 [9]
β-Mercaptoethanol	0.87 [17]	0.95[a] [14]	0.55 [9]
		0.72[b] [14]	
Thioglycolic acid	2.1 [This paper]		1.6 [9]
Thioacetic acid	2.5 [This paper]		–

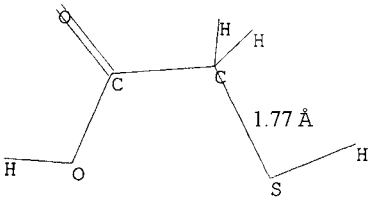
[a] Spectrophotometric.
[b] pH-stat.



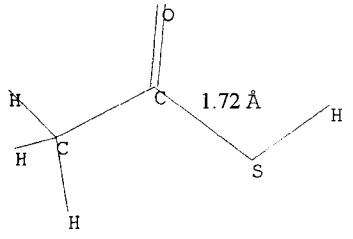
β-mercaptoethylamine



β-mercaptoethanol



Thioglycolic acid



Thioacetic acid

Fig. 4. Chemical structures of thiol compounds: β-mercaptoethylamine β-mercaptoethanol thioglycolic acid and thioacetic acid. The length of C–S bound calculated with use of CHEMWIN computer programme.

thioacetic acid≈thioglycolic acid<β-mercaptoethanol <β-mercaptoethylamine.

Obtained values of inhibition constants are compared with the inhibition constants of *Klebsiella aerogenes* urease at Hepes buffer, pH 7.75 with use of the ammonia selective electrode and differential form of the Michaelis–Menten equation (Table 2).

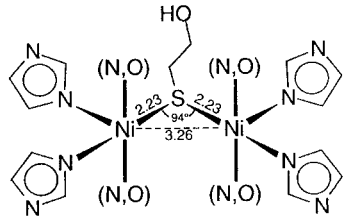


Fig. 5. Model of the bi-nickel center at the active site of β-mercaptoethanol-treated urease [25].

β-Mercaptoethylamine which has a positively charged β-amino group is the strongest inhibitor, β-mercaptoethanol which has a neutrally charged hydroxylate group is an inhibitor of a medium strength and thioglycolic acid which possesses an anionic carboxyl group is a poor inhibitor. This behaviour is a support for the presence of a negative charge at the urease active site. Thioacetic acid seems to be the poorest inhibitor among tested thiols (Fig. 3). The methyl group of thioacetic acid should not be a drawback for bounding to nickel ions. The hindrance might be an oxygen atom with two pairs of lone electrons. In the Fig. 4 are presented models of thiols with the length of C–S bound.

The pH dependence of the inhibition constant demonstrates that the deprotonated thiol acts as the inhibitor. In fact the actual inhibitory species for urease are thiolate anions and protoned thiols are not inhibitory active [9]. UV–Vis spectroscopy reveals that thiols bind directly to the active site nickel ions. Whereas the native enzyme has only a very weak UV–Vis spectrum, the addition of thiol compounds leads to spectroscopically detectable thiolate anion→Ni(II) charge transfer transitions [24]. Wang et al. [25] with the use of biophysical and spectroscopic analyses generated a model of bi-nickel center at the active site of jack bean urease. In the resting enzyme, the nickel ions are separated by ca. 3.5 Å distance. Externally added thiol (indicated as β-mercaptoethanol) bridges nickel ions and shrinks the distance between nickels to 3.26 Å (Fig. 5). It is highly probable that the same model can be applied for the other examined thiols.

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