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Thermal investigations of enzyme-catalyzed reactions for detection of heavy metals in the case of cadmium[1]

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Abstract

The present work gives the results of extensive calorimetric measurements to determine the inhibitory effect of cadmium ions on enzymatic urea hydrolysis. Three milligrams of urease and 10 mmol urea (kg solution)$^{-1}$ were found to be the optimum reaction conditions in 0.05 M HEPES buffer (pH 6.6). A correlation between the initial rate v_0 and the cadmium concentration in the ppm range can be demonstrated by determination of the initial slope of the calorimetric measuring curves. By determination of the enzyme kinetic data, a contribution to detailed information on the inhibitor mechanism of cadmium can be made with these results.

Keywords: Calorimetry; Enzyme; Enzyme kinetic data; Inhibitor; Heavy metal detection

1. Introduction

The inhibitory effect of heavy metals on enzyme activity is well known [1–3]. Urease has essential SH groups the blocking of which by heavy metals results in a decrease in its catalytic activity.

Thermal investigations of enzyme-catalyzed urea hydrolysis in the presence of cadmium should be used for quantitative detection of heavy-metal ions. However, the resulting possibility of analytic application of a thermal sensor requires detailed knowledge of suitable reaction conditions and thermokinetic data.

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The effect of the experimental conditions (buffer system, pH value) on the hydrolytic reaction was investigated in a previous work [4]. Within the scope of the present work the results of extensive calorimetric measurements for enzymatic urea hydrolysis are presented; the substrate concentration, the amount of enzyme and the cadmium concentration are varied.

A first investigation complex concentrates on the optimization of the amount of enzyme and substrate concentration to be used. Thermal investigations into the influence of cadmium on the enzyme-catalyzed reaction and its kinetic evaluation show a definite dependence of the initial rate v_0 on the cadmium content in the system. Experimental determination of the inhibition constants K_i and K_i' which are characteristic of the individual types of inhibitory effects and further thermokinetic data allow an assessment of the inhibition mechanism.

2. Experimental

The calorimetric measurements were performed in an isoperibolic LKB 8700 calorimeter produced by LKB Stockholm/Sweden at a temperature of 298.15 K. The specifications of the calorimetric measurements are fully detailed in [5]. Measurement data evaluation was done using an extensive computer program (to be published) which was developed by our institute.

In accordance with the details given by the manufacturer, the urease from jack bean (EC 3.5.1.5; produced by Serva) that we used had a specific activity of 260 U mg^{-1}.

A 0.05 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) solution with a pH value of 6.6 was chosen. In this reaction medium the formation of slightly soluble heavy-metal compounds was avoided to the greatest possible extent. Urea (p.a.) produced by Merck was used as a substrate.

In order to ensure a defined cadmium content in the system, an aqueous cadmium standard (Titrisol, Merck) with a content of 1 g l^{-1} Cd was used.

3. Results and discussion

Urea hydrolysis is catalyzed by urease. In the first step, the urea is converted into ammonium carbamate by urease. The further decomposition of ammonium carbamate into the respective products of hydrolysis depends on the buffer system chosen and the pH value [4].

For optimization of the reaction conditions for enzyme-catalyzed urea hydrolysis, some investigations in HEPES buffer with a pH value of 6.6 were carried out in order to determine the dependence of the initial rate v_0 of the reaction on the amount of enzyme m_E and substrate concentration $c_{urea}{}^0$.

Determination of the initial rate v_0 was carried out by differentiation of the linear initial range ($t < 110$ s) of the adiabatic temperature–time curve assuming that the yield is proportional to ΔT at the time t:

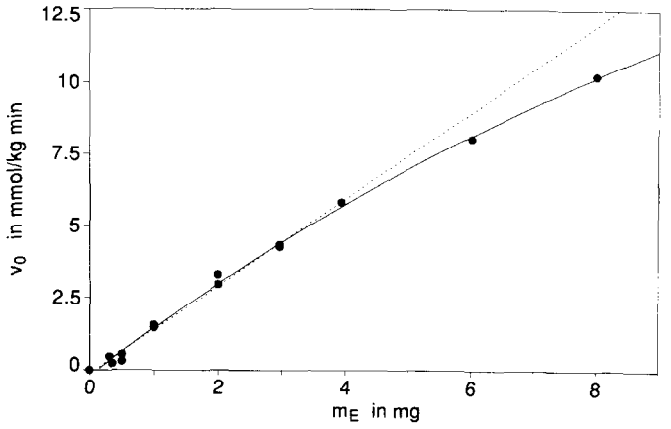


Fig. 1. Initial rate of enzymatic urea hydrolysis as a function of the amount of enzyme; ·····, kinetically controlled reaction sequence; $c_{urea}{}^0 = 10$ mmol (kg solution)$^{-1}$.

$$\Delta T = \Delta T_{max}(c_{urea}{}^0 - c_{urea})/c_{urea}{}^0 \quad (1)$$

where $c_{urea}{}^0$ is the urea concentration used, c_{urea} is the urea concentration at time t, ΔT is the adiabatic temperature difference at time t andΔT_{max} is ΔT with a complete yield.

For the initial rate v_0 follows

$$v_0 = -(dc_{urea}/dt) = (c_{urea}{}^0/\Delta T_{max})(d\Delta T/dt) \quad (2)$$

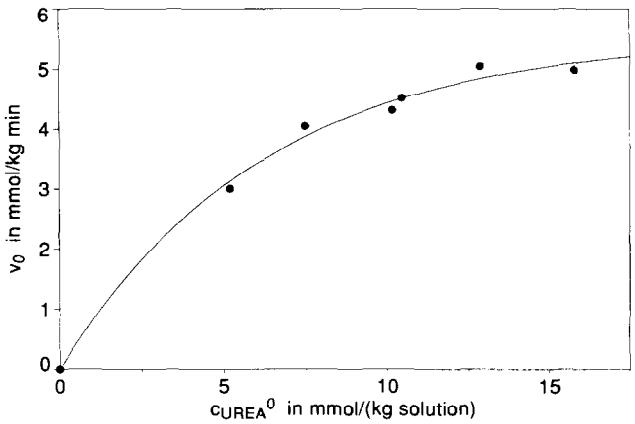


Fig. 2. Initial rate of enzymatic urea hydrolysis as a function of the urea concentration; $m_E = 3$ mg.

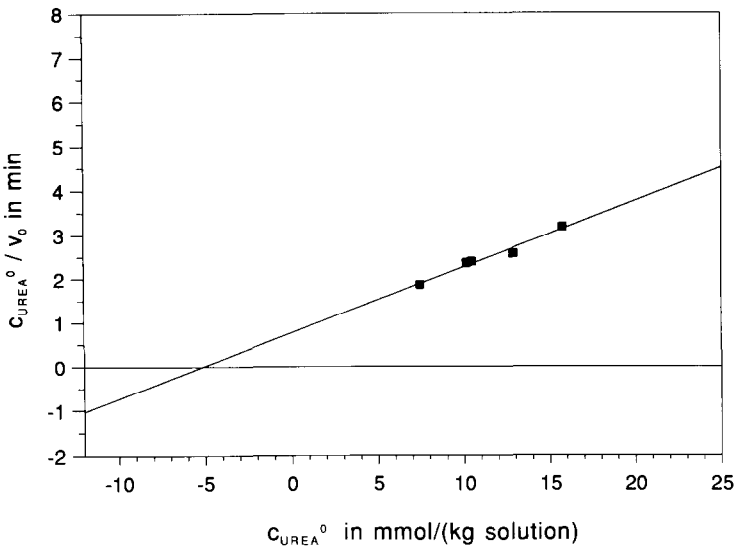


Fig. 3. Hanes-Woolf plot for determination of K_M.

Fig. 1 shows the transition of the kinetically controlled reaction sequence to a diffusion-controlled sequence with an increasing amount of enzyme m_E. The enzymatic reaction is the rate-determining step in kinetic control. The signal obtained depends on the amount of enzyme used.

Further increase in the amount of enzyme will result in a diffusion-controlled reaction mechanism. The enzyme activity of the system is so high that all substrate molecules which reach the enzyme are instantly converted. The linearity of the initial rate v_0 as a

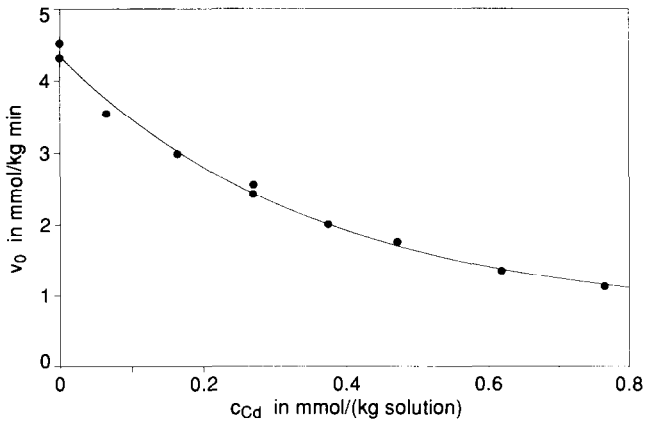


Fig. 4. Influence of cadmium on the initial rate of the enzymatic urea hydrolysis.

Table 1

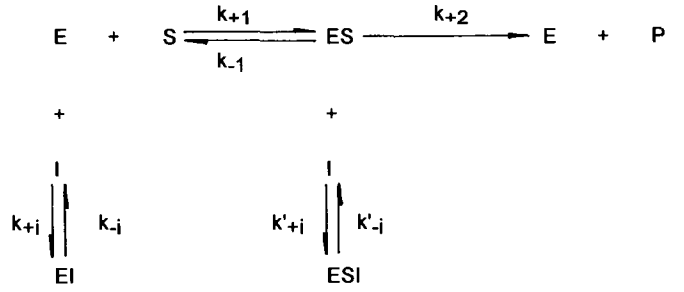
Molar reaction enthalpies $\Delta_R H_B$ of the enzymatic urea hydrolysis in dependence on the concentration of cadmium in the system

c_{Cd} in mmol (kg solution)$^{-1}$	$\Delta_R H_B$ in kJ mol^{-1}
0	−40.54
0.07	−40.81
0.16	−41.02
0.27	−40.64
0.27	−40.29
0.37	−41.98
0.47	−40.91
0.62	−41.78
0.76	−41.18

function of the substrate concentration used (c_{urea}^0) can no longer be ensured in this range.

Therefore, an amount of enzyme in the range of 1–4 mg (see Fig. 1) should be used to ensure a kinetically controlled reaction sequence. Three milligrams of urease were used for further investigations.

Experiments which were performed to determine the influence of the substrate con-



E - enzyme
S - substrat
ES - enzyme - substrat - complex
P - products
EI - enzyme - inhibitor - complex
I - inhibitor
ESI - enzyme - substrat - inhibitor - complex

inhibition constants:

$$K_i = \frac{k_{-i}}{k_{+i}} \qquad K'_i = \frac{k'_{-i}}{k'_{+i}}$$

Fig. 5. Possibility of inhibitor attack and determination of inhibition constants.

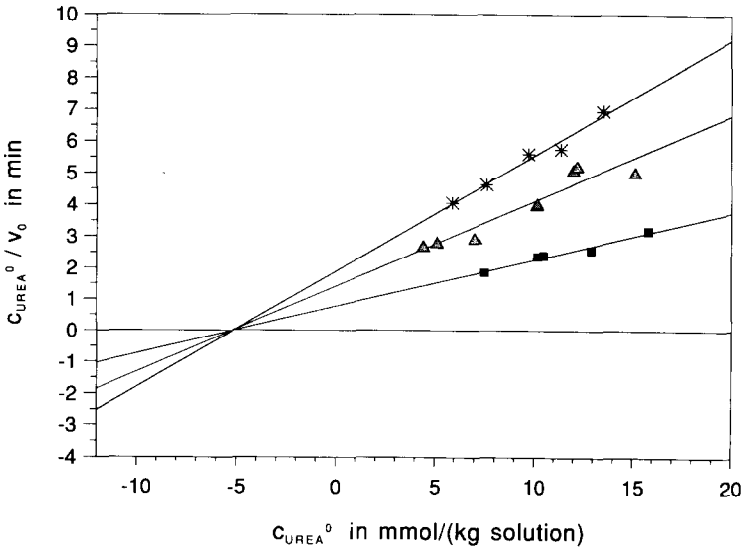


Fig. 6. Hanes-Woolf plot for calculation of enzyme kinetic data.; ■, non-inhibited reaction; △, addition of 2500 μg cadmium; ⁎, addition of 4500 μg cadmium.

centration $c_{urea}{}^0$ on the initial rate v_0 show an asymptotic approximation of v_0 to a limit above a urea concentration of 15 mmol (kg solution)$^{-1}$ (Fig. 2). This limit corresponds to the maximum reaction rate of the urease used in the respective reaction system. An increase in concentration of the substrate $c_{urea}{}^0$ beyond this value will not result in a considerable increase in the initial rate.

By variation of the urea concentration $c_{urea}{}^0$, the Michaelis constant K_M of the enzymatic urea hydrolysis could be determined under the chosen conditions. It gives the value of the substrate concentration, the reaction rate having a half-maximum value. Using the Hanes-Woolf diagram [6], $K_M = 5.1 \pm 0.8$ mmol (kg solution)$^{-1}$ (Fig. 3) was found. The resulting large error is due to the necessity of an extrapolation over a wide range to an ordinate value of zero.

Table 2

Enzyme kinetic data for the non-inhibited reaction and the cadmium inhibited reaction

	Non-inhibited reaction	Addition of 2500 μg cadmium	Addition of 4500 μg cadmium
v_{max} in mmol (kg min)$^{-1}$	6.7 ± 0.8	–	–
c_{Cd} in mmol kg^{-1}	–	0.27 ± 0.001	0.47 ± 0.001
$v_{max,i}$ in mmol (kg min)$^{-1}$	–	3.7 ± 0.6	2.7 ± 0.3
K_M in mmol kg^{-1}	5.1 ± 0.8	5.4 ± 0.7	5.1 ± 0.5
$K_i = K_i'$ in mmol kg^{-1}	–	0.33 ± 0.03	0.32 ± 0.04

The tests performed have shown an optimum amount of enzyme of 3 mg urease and an optimum urea concentration of 10 mmol (kg solution)$^{-1}$. The two values ensure the kinetic sequence of the reaction and a sufficient size of the measuring signal. The experimentally determined molar reaction enthalpy of the brutto reaction is $\Delta_R H_B = -40.0 \pm 0.9$ kJ mol^{-1} urea.

After optimization of the reaction conditions for thermal investigations of the enzyme-catalyzed urea hydrolysis, in further investigations cadmium in the range of 0.07 bis 0.76 mmol (kg solution)$^{-1}$ was added to the system. This corresponds to a maximum concentration of 80.2 ppm cadmium in the solution. Fig. 4 shows the correlation between the initial rate of the reaction and the cadmium concentration c_{Cd} in the system.

A comparison of the molar reaction enthalpies obtained for different cadmium content shows a good agreement of $\Delta_R H_B$ over the whole concentration range (Table 1). Consequently, only reaction kinetics is effected by the inhibitor.

In principle, there exist three methods [7] for the inhibitor to have an influence on the enzymatic reaction (Fig. 5). In the case of competitive inhibition there is a bond between the inhibitor and the free enzyme. As a result, an enzyme–inhibitor complex is formed. Inhibitor and substrate compete for the active site. An uncompetitive inhibition is characterized by an attack of the inhibitor on the enzyme–substrate complex with formation of a ternary enzyme–substrate–inhibitor complex. In the case of mixed inhibition, the inhibitor can have an influence on the reaction by bonding with the free enzyme and the enzyme–substrate complex.

The inhibition mechanism can be assessed by determination of the inhibition constants K_i and K_i' which are characteristic of the individual types of inhibitory effect. Using the Hanes-Woolf diagram (Fig. 6), the inhibition constants K_i and K_i' as well as the maximum reaction rates v_{max} and $v_{max,i}$, respectively, could be calculated (Table 2).

A non-competitive inhibitory effect of cadmium can be taken from the data summarized in Table 2. The non-competitive inhibition is a special case of the mixed inhibition.

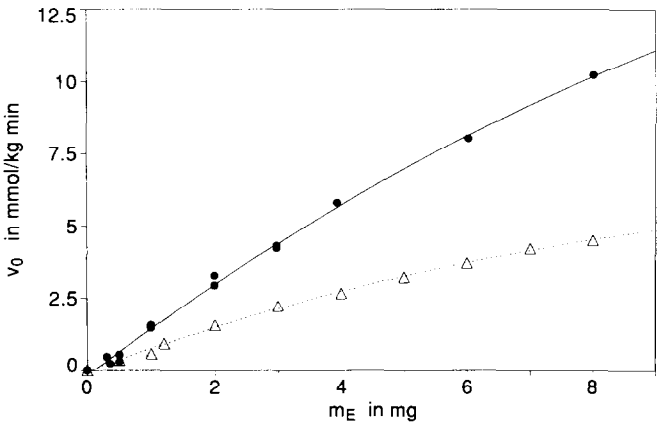


Fig. 7. Degradation of the initial rate of the enzymatic urea hydrolysis by cadmium inhibition effect; ■, $c_{Cd} = 0$ mmol (kg solution)$^{-1}$; △, $c_{Cd} = 0.27$ mmol (kg solution)$^{-1}$.

Equal quantity of K_i and K_i' is a characteristic feature of this special case. Cadmium has an influence on the maximum reaction rate v_{max}, but the K_M value keeps constant within the limit of error.

Fig. 7 shows the inhibitory effect of cadmium depending on the amount of enzyme used (m_E) compared with the non-inhibited reaction. Even by addition of large amounts of enzymes the reaction inhibition will continue to exist.

4. Summary

In this work the kinetics of the enzymatic urea hydrolysis in the presence of cadmium was investigated by means of calorimetric measurements. Optimum reaction conditions and knowledge of the inhibition mechanism of the heavy metal are a basic requirement of thermal investigations of the inhibitory effect of cadmium.

The investigations demonstrate a definite dependence of the initial rate of the enzymatic urea hydrolysis on the cadmium content of the system. Thermal investigations of the reaction can be used to quantify the cadmium content in the system and opens up new possibilities to apply a thermal sensor for heavy metal determination in the ppm range.

Acknowledgements

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