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Calorimetric investigations of urease inhibition by heavy metal ions[1]

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Abstract

The subject of this publication is calorimetric investigations on the inhibitor effect of heavy metal ions on the enzyme urease. The obtained results allow quantification of the inhibitors (Cd^{2+}-, As^{3+}-, Zn^{2+}-, Pb^{2+}-ions) via the initial reaction rate of enzymatically catalysed urea hydrolysis. The interpretation potential of the calorimetric measurements is underlined by the determination of the inhibiting mechanisms for the example of Cd^{2+}- and As^{3+}-ions, the findings on the time regime of the inhibition process, and the detection of heavy metal ions in the ppm range. The use of several different buffer substance revealed the influence of the latter on the intensity of heavy metal inhibition. This opens the path to both the selective analysis of heavy metals via pattern recognition and to the improvement of detection sensitivity. © 1998 Elsevier Science B.V.

Keywords: Calorimetry; Enzyme; Heavy metals; Inhibition; Kinetic data

1. Introduction

This paper presents calorimetric investigations on the inhibitor effect of heavy metals on the enzyme urease. The high sensitivity of urease toward heavy metal ions should provide a possibility for thermal detection. The initial reaction rate of enzymatically catalysed urea hydrolysis was used as a measured variable, since the heavy metal ions influence the activity and therefore the kinetics of the reaction. Conversion was complete for all conducted measurements, which was proven by a constant molar brutto reaction enthalpy. In addition to this analytic aspect, comparative investigations on the inhibition mechanism of heavy metal ions were performed for the examples of As^{3+}- and Cd^{2+}-ions. Further factors potentially influencing the inhibition, such as the buffer system used and the incubation time of the inhibitor agent, are the subject of this research work.

2. Experimental

The calorimetric measurements were performed in an isoperibolic LKB 8700 calorimeter. Before the reaction in the calorimeter, the investigated systems were thermostated to a constant temperature of 298.15 K. The experimental details have been given in Ref. [1].

In the standard arrangement, a solution consisting of buffer, heavy metal ions and urease was prepared in the calorimeter cell, with the urea solution in the ampoule. The reaction was initiated by breaking the ampoule.

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In accordance with the details given by the manufacturer, the urease (EC 3.5.1.5; produced by SERVA) that we used from jack bean had a specific activity of 260 U mg^{-1}. *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid (HEPES), citrat buffer and *bis*-(2-hydroxyethyl)-imino–*tris*(hydroxymethyl)-methane (*bis–tris*) were chosen. The urea (MERCK) concentration was varied over a range 5–16 mmol/(kg solution). In order to ensure a defined metal content (Cd^{2+}, Pb^{2+}, As^{5+}, Zn^{2+}) in the system, an aqueous metal standard (TITRISOL, MERCK) with a content of 1 g/l was used. In case of As(III), a solution of 1 g/l was produced by dissolving As_2O_3 in distilled water.

3. Results and discussion

Various heavy metals inhibit the activity of urease, thus influencing the reaction rate of the enzymatically catalysed urea hydrolysis. The bivalent ions of Zn, Cd and Pb, as well as the tri- and pentavalent ions of As, were selected as representatives of these inhibitors.

In accordance with the results published in Ref. [2], the brutto reaction equation of urea hydrolysis can be formulated as follows:

$$CO(NH_2)_2 + H_2O \overset{\text{urease}}{\rightarrow} NH_2COO^- + NH_4^+$$

$$\overset{H_2O}{\rightarrow} \text{products of hydrolysis}$$

The conversion of ammonium carbamate to the various products of hydrolysis depends on the buffer system or pH value selected [2]. In the present work, a uniform pH value of 6.6 was selected for all three buffer systems (HEPES, citrat and *bis–tris*). Depending on the buffer system used, the following molar brutto reaction enthalpies $\Delta_R H_B$ (Table 1) were determined for the conversion of urea to hydrolysis products.

The determined molar brutto reaction enthalpy $\Delta_R H_B$ is independent of the addition of heavy metal ions. The mechanism remains unchanged, and urea conversion is always complete; the heavy metal ions exclusively influence the kinetics of the urea hydrolysis. Therefore, the initial reaction rate v_0, according to Ref. [3], was evaluated as measured variable. To allow comparative analysis of the inhibitor effect of the different heavy metal ions, the determined initial reaction rate was converted to a relative enzyme activity expressed in percent.

Table 1
Molar brutto reaction enthalpy for the urea hydrolysis in the different buffer systems

Buffer	Concentration (mol/l)	$\Delta_R H_B$ (kJ/mol)
HEPES	0.05	−40.0±0.9
Bis–tris	0.05	−31.5±0.1
Citrat	0.5	−63.6±0.4

$$\text{relative enzyme activity} = \frac{v_0^*}{v_0} \times 100\% \qquad (1)$$

v_0 is the initial reaction rate of the non-inhibited reaction; and v_0^* the initial reaction rate of the inhibited reaction.

Fig. 1–4 represent the dependence of this relative enzyme activity on heavy metal ion concentration;

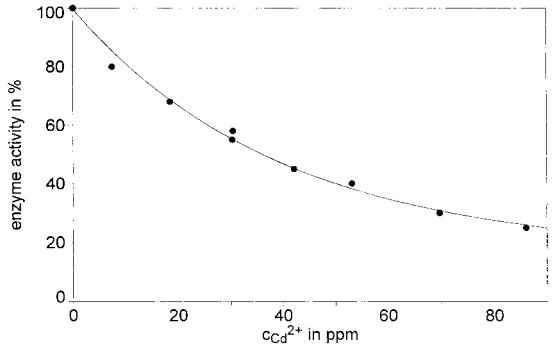


Fig. 1. Inhibitor effect of Cd(II)-ions.

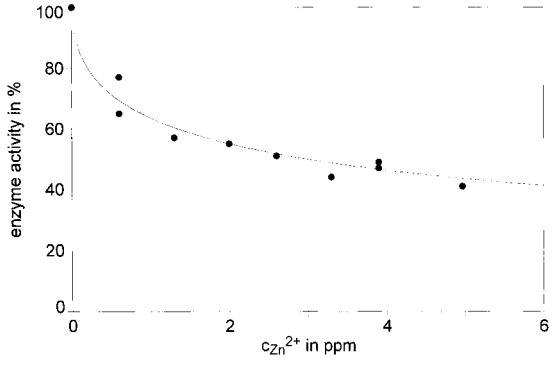


Fig. 2. Inhibitor effect of Zn(II)-ions.

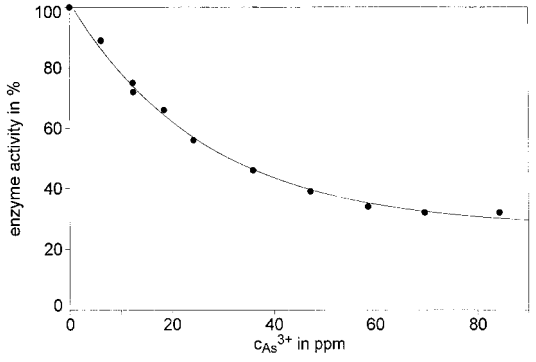


Fig. 3. Inhibitor effect of As(III)-ions.

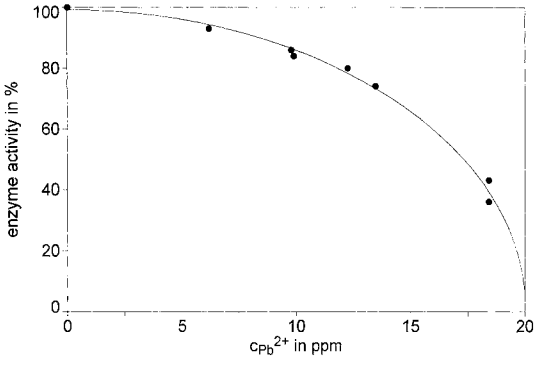


Fig. 4. Inhibitor effect of Pb(II)-ions.

they prove that calorimetric detection of the heavy metals in the ppm range is possible. It is evident that there are pronounced differences in the intensity of the inhibitor effect of various heavy metal ion species investigated. The I_{50}-values of the heavy metal ions, which indicate the heavy metal ion concentration that provokes a 50% inhibition of the enzyme, follow the order $Zn^{2+}<As^{3+}<Cd^{2+}$. The I_{50}-value of the Pb(II)-ions cannot be included in this series since these ions react with the enzyme under precipitation. In contrast to the clear inhibition of urease by As(III)-ions, test measurements of the inhibitor effect of As(V)-ions at a concentration of $c_{As(V)}=31$ ppm did not show any reduction of the initial rate of enzymatically catalysed urea hydrolysis. Due to the very different degree of toxicity of different valencies of arsenic, such analyses can provide an unambiguous differentiation between the different valencies if the total arsenic content is known first.

Table 2
Arrangement of the reactants

Arrangement	Ampoule	Calorimeter cell
Standard △	Urea HEPES-buffer	Urease Cd^{2+}-ions HEPES-buffer
Variant ●	Urea Cd^{2+}-ions HEPES-buffer	Urease HEPES-buffer

In addition to the above analytic aspects, the described calorimetric measurements also allow to investigate the time regime of the inhibition. To this end, the arrangement of the reactants in the calorimeter (Table 2) was varied for the example of Cd^{2+}-ions.

The standard arrangement allows a reaction between enzyme and heavy metal in the thermostating phase. In contrast, the second variant provides for the heavy metal ions to be added to the enzyme only when the reaction starts. Due to the possibility, in the standard arrangement, of a reaction between inhibitor and enzyme prior to the start of the reaction as such, the enzyme activity is reduced more strongly than in the variant arrangement (Fig. 5). Thus, the establishment of the dissociation equilibrium between the Cd^{2+}-ions and the enzyme or the enzyme substrate complex is time-dependent. On the other hand, the fact that urease inhibition also takes place when substrate and enzyme are added simultaneously shows that the inhibition rate is comparable to the initial rate of the enzymatically catalysed urea hydrolysis.

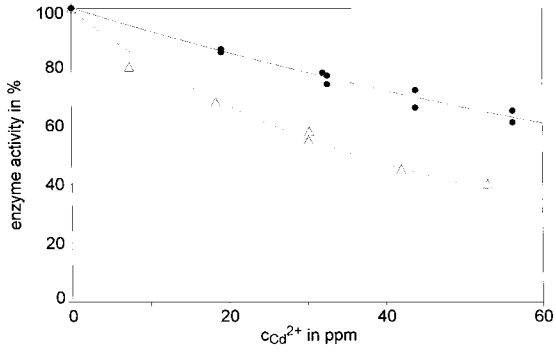


Fig. 5. Variation in the arrangement of the reactants – time regime of the inhibition. △: standard; ●: variant.

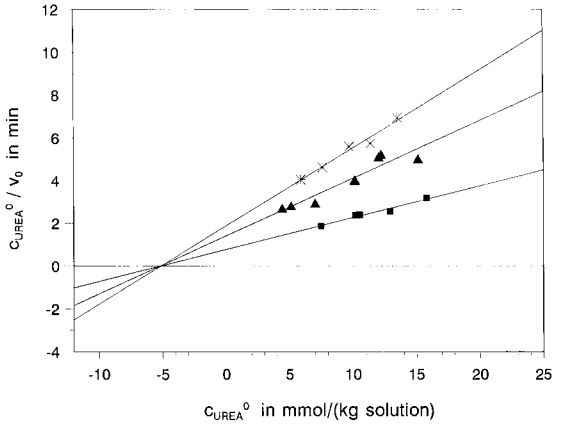


Fig. 6. Hanes–Woolf plot for the inhibition of Cd(II)-ions. ■ $c_{Cd(II)}$=0 mmol/(kg solution); △: $c_{Cd(II)}$=0.27 mol/(kg solution); *: $c_{Cd(II)}$=0.47 mmol/(kg solution).

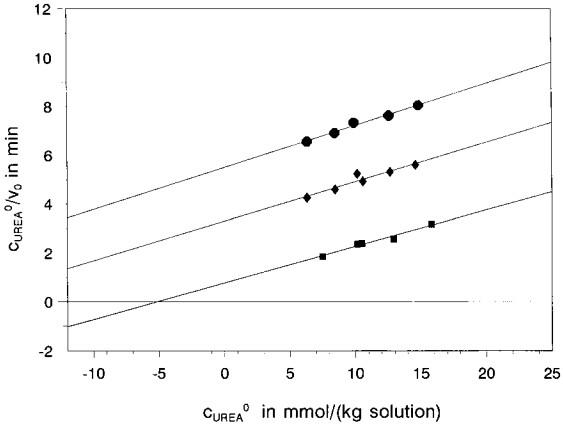


Fig. 8. Hanes-Woolf plot for the inhibition of As(III)-ions. ■: $c_{As(III)}$=0 mmol/(kg solution); ◆: $c_{As(III)}$=0.33 mmol/(kg solution); ●: $c_{As(III)}$=0.66 mmol/(kg solution).

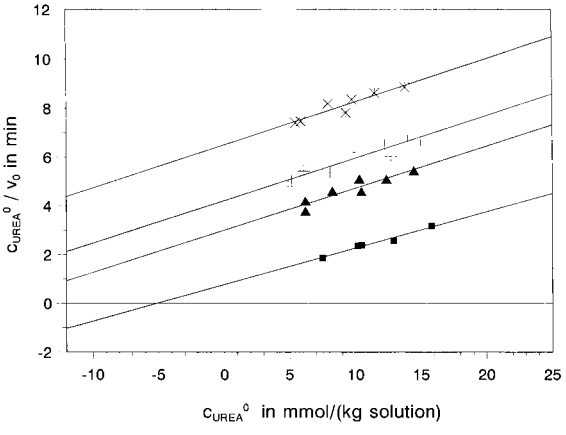


Fig. 7. Hanes-Woolf plot for the inhibition of As(III)-ions. ■: $c_{As(III)}$=0 mmol/(kg solution); △: $c_{As(III)}$=0.25 mmol/(kg solution); +: $c_{As(III)}$=0.48 mmol/(kg solution); *: $c_{As(III)}$=0.78 mmol/(kg solution).

With regard to the mechanism of the inhibitor effect of heavy metal ions on enzymatically catalysed urea hydrolysis, comparative investigations were carried out for the example of Cd^{2+}- and As^{3+}-ions. The linearised version of the Michaelis–Menten relation according to Hanes–Woolf [4] (Fig. 6–8) was used to determine the inhibition mechanisms. As a result, it was shown that the Cd^{2+}-ions inhibit the urease activity on the basis of a non-competitive mechanism, whereas a partially competitive mechanism [5] was found for the As^{3+}-ions. The time dependence of the inhibitor effect does not exert an influence on the inhibiting mechanism of the cadmium ions. The inhibiting mechanism of this heavy metal ions was discussed in detail in Ref. [3].

Figs. 7 and 8 show the pattern of the competitive inhibition mechanism of the As(III)-ions. The fact that the straight lines are parallel indicates that the As^{3+}-ions do not influence the maximum reaction rate v_{max} of the enzyme (slope of line=$1/v_{max}$). With increasing inhibitor concentration, however, the affinity of the substrate to the enzyme is reduced. In view of the signs of a partially competitive inhibition by As(III)-ions (see Fig. 3, no further reduction of the initial reaction rate above $c_{As(III)}$=0.78 mmol/(kg solution)), the measured values were analysed in greater detail using a Dixon representation [6] (Fig. 9). A deviation from the straight line was established at high inhibitor concentrations. This deviation is the result of a partially competitive inhibition [7].

The results presented so far were obtained in an HEPES buffer. Investigations in two other buffer systems (citrat buffer and *bis–tris* buffer) have illustrated the impact of this reaction parameter on the inhibitor effect of the selected heavy metal ions. Fig. 10 shows how the interaction between the heavy metal ions and the buffer substance influences the intensity of inhibition. The influence of the buffer system was found to be most pronounced with zinc ions. The reduced inhibition effect of the zinc ions in

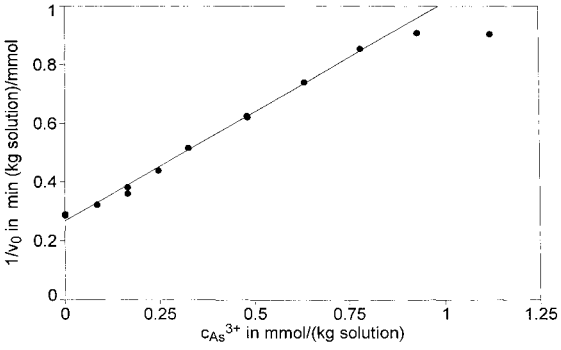
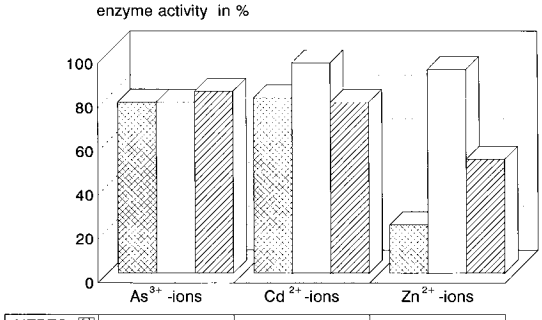


Fig. 9. Dixon plot for the inhibition of As(III)-ions.



	As^{3+} -ions	Cd^{2+}-ions	Zn^{2+} -ions
HEPES	78	80	22
Citrat	78	96	93
Bis- Tris	83	78	52

Fig. 10. Influence of the buffer system.

the citrate buffer indicates a strong tendency toward complex formation for zinc.

Thus, on the one hand, the selection of suitable, chemically inert buffer systems allow to improve the sensitivity of the detection method; on the other hand, the complex-forming properties of various buffers (e.g. the citrate buffer) allow the selective complexion of individual heavy metals. In addition to the determination of sum parameters, this allows the detection of certain heavy metals by the principle of pattern recognition.

4. Summary

Using the thermal measurement principle, the inhibitor effect of the bivalent ions of cadmium, lead and zinc, as well as tri- and pentavalent arsenic ions, on the enzyme urease was investigated. Results show a clear dependence of the initial reaction rate of urease-catalysed urea hydrolysis on the inhibitor concentration, which allows the thermal detection of the heavy metals in the ppm range. The characteristic I_{50}-values of the heavy metal ions increase in the order $Zn^{2+}<As^{3+}<Cd^{2+}$. In contrast to trivalent arsenic ions, their pentavalent equivalents do not inhibit the enzyme in the comparable concentration range.

Extensive calorimetric measurements provided insight into the mechanism of inhibition by the selected heavy metals. In each case, the heavy metal ions exclusively influenced the time regime of the reaction, while substrate conversion was always complete. Comparative investigations on the inhibition mechanism were conducted for the example of Cd^{2+}- and As^{3+}-ions. While Cd^{2+}-ions inhibit urease activity by a non-competitive mechanism, a partially competitive mechanism was found for As^{3+}-ions. Varying arrangement of the reactants in the calorimeter allowed conclusions on the time regime of the inhibition.

The potential of a suitable selection of the buffer system is discussed with respect to the increase of the sensitivity as well as pattern recognition in the detection of heavy metal species by calorimetric methods.

Acknowledgements

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