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The Enzymatic Determination of Mercury and Copper Using Acid Urease. The Effects of Buffers

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Abstract. We report on a quick and simple test based on enzyme inhibition for the detection of mercury and copper using free acid urease coupled to an optical sensor system. Lipophilized Nile Blue was incorporated in plasticized poly(vinyl chloride) (PVC) to produce an ammonium-sensitive layer with a thickness of around 4 μm. The layer was fixed on one side of a disposable cuvette. A solution of buffer, enzyme and heavy metals was placed into the thermostated cell. Enzymatic hydrolysis was started upon addition of urea and the formation of ammonium was monitored. Mercury and copper were the strongest inhibitors; for this reason the inhibitory efficiency of these metals was examined in citrate, acetate and trismaleate buffers. The cuvette test was most sensitive and selective for mercury in a citrate buffer. The limit of detection for mercury(II) ions was as low as 1 μg/L. Copper ions do not interfere because of complexation by citrate. The inhibitory effects of metal combinations on the activity of acid urease and the effects of optimum pH of the enzyme and the transducer on the dynamic range of the cuvette test are presented.

Key words: mercury, copper, acid urease, ammonium optode.

The need for faster and more cost-effective methods for environmental monitoring has led to a variety of environmental field analytical methods, such as miniaturized laboratory methods, field test kits, and chemical (bio)sensors. Because of their unique characteristics biosensors might be exploited to fill specific niche applications in the environmental monitoring area [1]. Biosensors for the determination of phenols [2], formaldehyde [3], nitrates [4], pesticides [5,6], biological oxygen demand (BOD) [7–9] and heavy metals [10–12] have been reported.

Heavy metals are known for their toxicity in the environment, in water, food and air. Because biological components such as enzymes are highly sensitive and selective, the enzyme acid urease was chosen for the determination of mercury and copper. Enzyme systems using ascorbate oxidase and alkaline phosphatase [13], glucoseoxidase [14], urease [15,16], invertase [17] and several dehydrogenases [18], have been reported. Danzer *et al.* [19] have combined three enzyme electrodes (acetylcholinesterase, acid and alkaline phosphatase) for pesticide and heavy metal screening using selected chemometric methods. The toxicity of metals towards fish and luminescent bacteria [20] was also used for a determination of heavy metals. Different transducer systems have been employed: (a) optical [15,16,21], (b) electrochemical [18,19] and (c) ISFET (ion-selective field effect transistor) [6].

We made use of free acid urease and an ammonium-sensitive optode to measure ammonium ion produced in the enzyme-catalyzed reaction. Because measurements were performed at the optimum pH of the enzyme and the optical transducer, trace levels of mercury and copper could be detected.

Experimental

Apparatus

Absorbance measurements were performed using a Shimadzu UV 2101 PC Scanning Spectrophotometer. The optode layer was fixed in a $1 \times 1\,cm^2$ disposable polythene cuvette (Brand, Germany)

thermostated at 25 °C in a water bath. Absorbance was measured at 665 nm by using an ammonium-sensitive layer.

Reagents

Ammonium chloride, tris(hydroxymethyl)aminomethane, and maleic acid were purchased from Aldrich (Steinheim, Germany). Nile Blue A (no. 72480), poly(vinyl chloride) (PVC) (high molecular weight). 2-nitrophenyloctylether (NPOE), potassium tetrakis(4-chlorophenyl)borate(PTCB), nonactin (no. 74155), mercury nitrate monohydrate and cupric nitrate trihydrate were obtained from Fluka, Chemie AG (Buchs, Switzerland). Citric acid, disodium hydrogen phosphate and sodium acetate were from Merck (Darmstadt, Germany). Polyester film, Mylar, type GA 10, was from Du Pont (Bad Homburg, Germany), and acid urease (26.6 U/mg) from Asahi Chemical Co. (formerly Jozo Co. Ltd.) (Shizuoko, Japan).

Standard Solutions

0.1 M citrate buffer of pH 4.6, 0.1 M sodium acetate buffer of pH 4.6, and 0.1 M trismaleate buffer of pH 6 were prepared according to the buffer table of Ciba Geigy [22].

Ammonium (NH_4^+) solutions were prepared by dissolving ammonium chloride in the respective buffer. The concentration of NH_4^+ ion was calculated by using the Henderson-Hasselbach equation which, at various pH-values and 25 °C, is:

$$pH = pK + \log[NH_3]/[NH_4^+] \quad (1)$$

The standard solution of urea was 1 M. Solutions of heavy metals were prepared by diluting 10^{-4} M stock solutions with the respective buffer. The concentration of the metal solutions was proven by atomic absorption spectroscopy (AAS).

Ammonium Transducer

The ammonium-sensitive layer contained a neutral NH_4^+-selective ionophore (nonactin), a neutral proton-selective chromo-ionophore (ETH 5294) (lipophilized Nile Blue) [23] and a lipophilic counterion [(potassium tetrakis (4-chlorophenyl) borate, PTCB)]. The "cocktail" consisted of 2 mg lipophilized Nile Blue, 5 mg nonactin, 2 mg PTCB, 120 mg PVC and 244 mg NPOE. All components were dissolved in freshly distilled THF and spread over a 175 µm polyester film to create a layer with thickness of around 4 and 8 µm (as calculated) after solvent evaporation. Before measurement, the indicator was protonated by placing the sensor layer in a solution of 0.1 N HCl (preconditioning).

Transducer Principle

The sensor was based on ion exchange, i.e. the release of a proton from a dye caused by the transport of an ammonium cation into the polymer. The sensor layer contained a neutral NH_4^+ – selective ionophore I (nonactin), a neutral H^+ ion-selective chromoionophore C (ETH 5294), and a lipophilic counterion (PTCB). The chromoionophore drastically changed its absorption spectrum upon protonation and formed a charged complex (CH^+). The ammonium ions in the sample solution were transported into the layer by the ionophore I to form a charged complex either (INH_4^+). Addition of the lipophilic counterion defined the total concentration of the positively-charged species in the layer. The ammonium ions were exchanged for the hydrogen ions of the chromoionophore C. As a result, the layer turned pink. Figure 1 gives a schematic of the process.

pvc	*water*
CH^+ (blue)	NH_4^+
I	X^-
PTCB	

(a)

pvc	*water*
C (pink)	H^+
INH_4^+	X^-
PTCB	

(b)

Fig. 1. Schematic outline of process (a) before and, (b) after exposure to ammonium (NH_4^+)

Test Principle

The ammonium-sensitive layer was glued to one wall of a $3.5 \times 1\,cm^2$ cuvette [12].

In order to measure 100% enzyme activity, solutions of 2.9 ml buffer and 0.2 ml acid urease were placed in the cuvette. To measure inhibition by means of metal ions, solutions of 2.8 ml buffer, 0.2 ml acid urease and 0.1 ml heavy metal were added. The total volume in the cuvette was 3.1 ml. Upon the addition of 0.4 ml standard urea solution, acid urease catalyzes the hydrolysis of urea according to:

$$(H_2N)_2CO + 3\,H_2O \xrightarrow{\text{acid urease}} 2NH_4^+ + HCO_3^- + OH^- \quad (2)$$

The initial rate of each uninhibited and inhibited enzyme reaction was calculated as the change of absorbance per 120 seconds. Measurements were performed immediately after the addition of metal solution, to overcome the sensitivity loss due to fast deactivation of acid urease by high metal concentrations ($>10^{-5}$ M). The ratio between the slopes of uninhibited ($\Delta E/s$) and inhibited from ($\Delta E'/s$) was expressed as % inhibition (x), according to:

$$x(\%) = (100 - 100\Delta E/\Delta E') \quad (3)$$

Figure 2 shows the inhibition of acid urease by Hg(II) ions plotted as changes of absorbance with time.

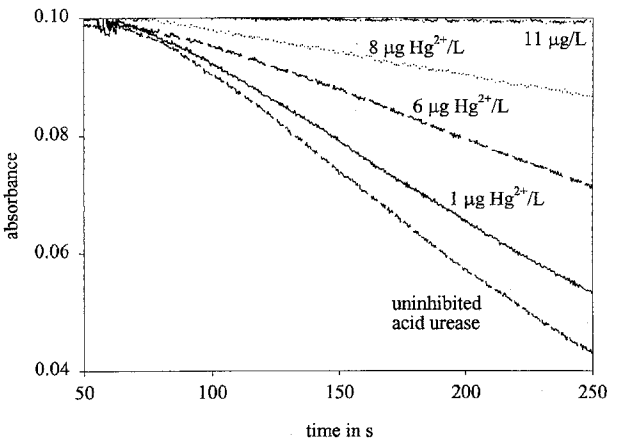

Fig. 2. Inhibition of acid urease by 1, 6, 8 and 11 µg/L mercury (II), plotted as change of absorbance with time

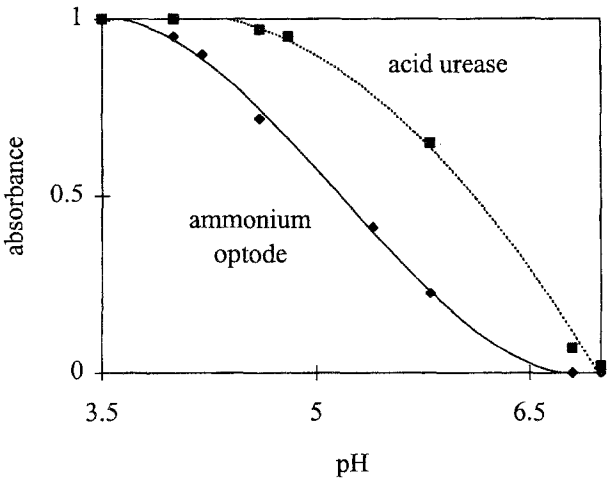

Fig. 3. Effects of pH on the response of the ammonium-sensitive layer (solid line) and the activity of acid urease (dotted line). The responses of the sensor and the enzyme activity were measured as changes in absorbance

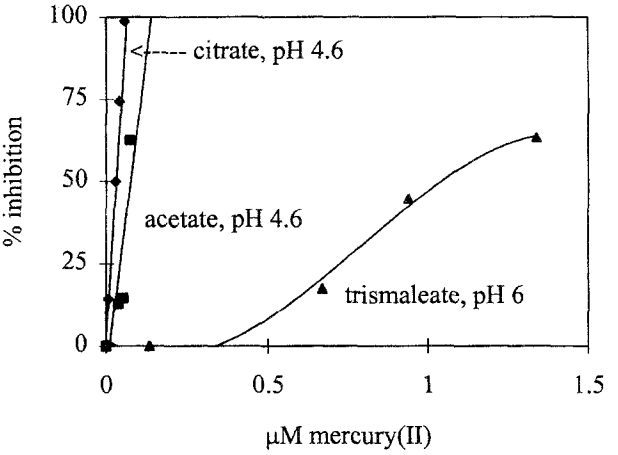

Fig. 4. Calibration graphs of acid urease inhibited by Hg(II) ions in acetate (pH 4.6), citrate (pH 4.6) and trismaleate buffer (pH 6)

Table 1. Limits of detection (*LOD*), linear range and metal concentration giving 50% inhibition (I_{50}) are listed in order to compare the inhibitory efficiency of Hg(II) and Cu(II) in acetate, citrate and trismaleate buffers

Heavy metal	Buffer	LOD in μM	LOD in μg/L	Linear range in μg/L	I_{50} in μg/L
Hg(II)	acetate, pH 4.6	0.04	8.0	8.0–24.1	16.0
Hg(II)	citrate, pH 4.6	0.005	1.0	1.0–20.1	5.7
Hg(II)	trismaleate, pH 6	0.5	100.0	118.4–281.1	200.0
Cu(II)	acetate, pH 4.6	5.0	300.0	300.0–2880.0	1280.0
Cu(II)	citrate, pH 4.6	–	–	–	–
Cu(II)	trismaleate, pH 6	40.0	2600.0	2600.0–7040.0	–

Results

Optimum pH of Transducer and Enzyme

Figure 3 shows the titration curve of the NPOE plasticized NH_4^+ sensor (pK = 5.2) and the effect of pH on the activity of acid urease. As can be seen from Fig. 3 the pH range suitable for measurement of NH_4^+ ions was pH 3.8 to pH 6.5. The optimum sensitivity of the transducer, which was referred to as the slope of the linear regression curve, was obtained in the range of pH 4.6 to pH 6. For this reason pH 4.6 was stated to be the optimum pH of the optical transducer. The use of the cuvette test at pH 4.6 made it possible to measure over 2 pH units. The optimum pH of the enzyme was between pH 3.5 and 4.6, which was a narrow range compared to the broad optimum range of urease from jack beans (from pH 6 to 7.5).

Reagent Concentration

The cuvette test was calibrated at fixed concentrations of substrate and enzyme. The optimum enzyme activity for the inhibition reaction was 1.5 U/cuvette test. Enzyme activities higher than 3 U/test showed less inhibition for the same heavy metal concentration, which is a well-known fact reported by several authors. Lower enzyme activities resulted in stronger inhibition, but suffered from decreased reaction rate and dynamic range further affected by the poor resolution of the ammonium sensor at low NH_4^+ concentrations. 0.1 M urea/cuvette was used in order to measure the reaction kinetics under substrate saturation.

Inhibition by Mercury

The inhibition of acid urease by Hg(II) using citrate, acetate and trismaleate buffers is shown in Fig. 4. All values are mean values. Figures of merit are listed in Table 1. Acid urease was 100 fold stronger affected by Hg(II) in citrate and acetate buffers of pH 4.6 than in a maleate buffer of pH 6, which was not an effect of the buffer, but was due to the increased enzyme activity and the poor response of the NH_4^+ sensor at pH 6. While a linear relation between relative inhibition (%) and the Hg(II) concentration was observed in citrate and acetate buffers, a sigmoidal inhibition curve was found for Hg(II) in a maleate buffer. The mercury concentration resulting in 50% enzyme inhibition (I_{50}) was 0.03 μM (5.7 μg/L) in a citrate buffer, 0.08 μM

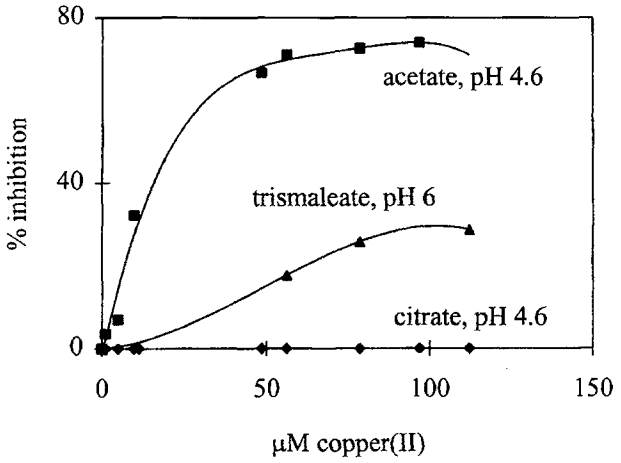

Fig. 5. Inhibition of acid urease in acetate (pH 4.6), citrate (pH 4.6) and trismaleate buffers (pH 6) as a plot of % inhibition against copper (II) concentration

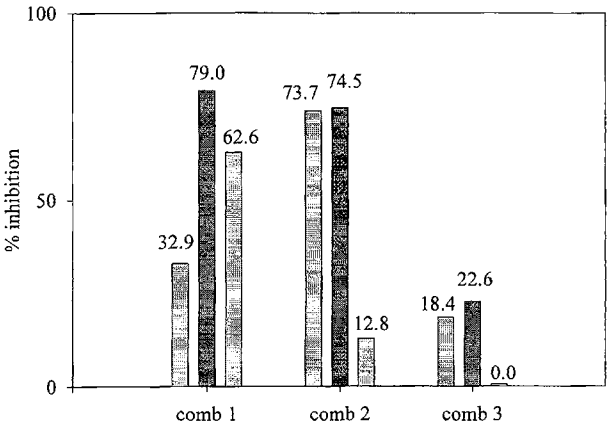

Fig. 6. Effects of combinations of Cu(II) and Hg(II) on the activity of acid urease: comb 1:9.7 µM Cu(II) and 0.07 µM Hg(II); comb 2: 29 µM Cu(II) and 0.04 µM Hg(II); comb 3: 100 µM Cu(II) and 0.006 µM Hg(II). The first column of each triplet corresponds to the inhibition by Cu(II), the second to the inhibition by both metals, and the third to the inhibition by Hg(II), respectively.

(16.0 µg/L) in an acetate buffer and 1.10 µM (0.2 mg/L) in a trismaleate buffer, respectively.

Inhibition by Copper

In Fig. 5 percent inhibition is plotted against Cu(II) concentration. The inhibition of acid urease by copper is strongest in an acetate buffer. Only a small effect was measured in a maleate buffer, but no inhibition was found in a citrate buffer [25]. The dynamic (linear) range of the test was 5–45 µM Cu(II) in an acetate buffer and 40–110 µM Cu(II) in a maleate buffer. % inhibition, LOD's and I_{50} values for Cu(II) in acetate, citrate and trismaleate buffers are listed in Table 1.

Inhibition by Other Metals

The inhibitory effect of 1 mM Fe(III), 1 mM Pb(II), 1 mM Cd(II), 1 mM Ni(II) and 1 mM Zn(II) on acid urease was examined. Inhibition was in all cases less than 5%, which is within the standard deviation. Because heavy metals other than mercury and copper are less effective, their inhibitory efficiency on acid urease was not studied in detail.

Combinations of Cu(II) and Hg(II)

The relative inhibition (%) of acid urease by a binary combination of Hg(II) and Cu(II) was compared to the inhibition by each single metal, and is shown in Fig. 6. The combination of copper and mercury in an acetate buffer resulted in an addition of effects (see comb 1 and comb 2 in Fig. 6). In the case of 0.006 µM Hg(II) and 100 µM Cu(II) in a citrate buffer (comb 3), the inhibition was almost exclusively due to the stronger inhibitor mercury. In contrast to previous studies [12], no synergistic effect of Cu(II) ions on the activity of acid urease was observed.

Reproducibility

Uninhibited and inhibited enzyme reactions were alternately measured. The average standard deviation s of all measurements was 5.4%.

Operational lifetime

The optode layer fixed in the cuvette was used 10–15 times before being replaced. The layer was regenerated with 0.1 N HCl after each measurement.

Storage lifetime

The lifetime was limited by the volatility of the plasticizer. The layers were stored at room temperature and remained fully active for three months.

Discussion

The optical cuvette test for the determination of mercury and copper is simple, and is thus suitable for

quick outdoor measurements. The optical and enzymatic parts of the cuvette test are separated. Components of the optical transducer were embedded in a polymer to create a thin layer. The solid state sensor was fixed on one wall of the cuvette. The enzymatic reaction, however, took place in solution. We used free (not immobilized) acid urease to avoid problems with poor enzyme stability and, hence, with the reproducibility of measurements.

The inhibition of acid urease by mercury, copper and other heavy metals was due to the binding of metals to the sulfhydryl groups of the enzyme. Heavy metals that show the highest affinity to the sulfur groups and form the most stable sulfides are the most toxic to acid urease. This phenomenon is true for most SH-containing enzymes [12].

The limit of detection (LOD) for Hg(II) ions were 8 µg/L in acetate, 1 µg/L in citrate and 0.1 mg/L in trismaleate buffer. The LOD of mercury in trismaleate buffer was 100 times higher than in citrate buffer. The low sensitivity of the test in trismaleate buffer was due to the poor sensitivity of the ammonium transducer at pH 6. The enzymatic reaction which caused the production of ammonium due to the hydrolysis of urea was monitored by the optical transducer upon the deprotonation of the immobilized indicator. The ammonium sensor was characterized by its pK, which was 5.2 and indicated the pH-value leading to 50% protonated (CH^+) and 50% unprotonated chromoionophore (C). As can the seen from Fig. 3, at pH 6 only 15% of the chromophore was present in its protonated form and was thus able to sense the enzymatic reaction. At pH 4.6, however, 80% of the chromophore was protonated. Therefore, a change of 2 pH-units (from pH 4.6 to pH 6.5) as a result of enzymatic reaction could easily be monitored. Measurements using trismaleate buffer of pH 6 and urease from jack beans [12] (optimum pH 6–7.5), instead of acid urease (optimum pH < 4.6) showed identical calibration curves for mercury. Hence, the optimum pH of the enzyme played a less important role in the sensitivity of the cuvette test. This might be due to the fact that the enzyme was replaced after each measurement and was not used continuously. Obviously, it was not the choice of enzyme that rendered the cuvette test highly sensitive, but the match of optimum pH of the ammonium-sensitive layer and the optimum pH of the enzyme.

The lowest copper concentrations possible to detect were 0.3 mg/L in acetate buffer and 2.6 mg/L in trismaleate buffer (see Table 1), e.g. the cuvette test was 8 times more sensitive in an acetate buffer than in a trismaleate buffer. Measurements in buffer of pH 6 are less sensitive due to the low sensitivity of the ammonium sensor at higher pH values. As explained before, it is important to consider not only the optimum pH of the enzyme, but also the pK of the transducer in order to achieve as low detection limits as possible.

Furthermore, the inhibition is also affected by the kind of buffer and its complexation ability [24, 25]. No inhibition of acid urease by Cu(II) was observed in a citrate buffer which was due to the complexation of copper by citrate. Thus, the cuvette test presented here makes possible the selective detection of mercury in presence of copper ions. The most stable complexes are formed by cations with high loading and small ionic radius. The ionic radius of copper is 0.072 nm and the ionic radius of mercury is 0.110 nm, respectively. Copper is bonded to the oxygen of the carboxylic group in a moderately acidic environment while mercury was not affected by citric acid and did not form a complex [26, 27]. As a result, Hg(II) concentrations as low as 1 µg/L Hg(II) could be detected selectively in the presence of Cu(II).

Conclusion

The optical cuvette test presented here makes use of free acid urease and lipophilized Nile Blue incorporated in a sensor layer. The use of an immobilized indicator allows the repeated measurement of produced ammonium under the same conditions resulting in a high reproducibility of the test. Furthermore, NH_4^+ was measured directly and selectively by employing a selective ion carrier (nonactin). Preparation of the optode layers was very rapid; similar performances were obtained with different optode layers. In any case, it is important to note that a perfect reproducibility of the optode layer was not an important feature, since a calibration curve had to be obtained before any quantitative analysis. The optical cuvette test using citrate buffer of pH 4.6 makes possible the detection of 1 µg/L Hg(II). Moreover, Cu(II) ions do not interfere, due to complexation by the buffer. The highly-sensitive and selective determination of Hg(II) ions is potentially suitable for the determination of Hg(II) in drinking water, since the standard for Hg(II) in drinking water set by WHO is 2 µg/L [28]. The detection limits for Cu(II) in acetate

buffer are in good agreement with the levels of copper in drinking water (1.5 mg/L), in fruit juices and alcoholic liquors (5–30 mg/L) recommended by the Swiss government and therefore would allow real test measurements.

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