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CONDUCTIVITY MEASUREMENT OF THE INHIBITORY EFFECT OF HEAVY METAL IONS ON IMMOBILIZED UREASE

PANOTE THAVARUNGKUL[1], PROESPICHAYA KANATHARANA[2], NATTAWUT NIPANUN[2], JITSANU PETCHARAT[2] AND PUNNEE ASAWATRERATANAKUL[3]

[1]Department of Physics, [2]Department of Chemistry, [3]Department of Biochemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Songkla 90112, Thailand.

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ABSTRACT

The inhibitory effect of heavy metals on the enzymic reaction of urease was determined by comparing the conductivity peaks of sample solutions resulting from hydrolysis of urea into charged products, before and after the introduction of inhibitors. A linear relationship between percentage of inhibition and concentration of heavy metal ions was observed with concentrations in the range of 10^{-4}-10^{-3} M for copper(II) and 10^{-6}-10^{-5} M for mercury (II). The enzyme column can be regenerated by the passage of a solution of NaI followed immediately by EDTA.

INTRODUCTION

The use of enzymes for analytical purpose has in the past few decades acquired considerable importance. Enzymes, as catalysts, make excellent analytical reagents due to their selectivity, and sometimes specificity. They are frequently used to determine the concentration of their substrates. The activity of enzymes can decrease in presence of some substances and such inhibitory effects have been used to detect toxic pollutants such as pesticide residues [1,2] and heavy metal ions [3-5].

Heavy metals are of significant environmental importance due to their toxicity, and their effects have been described [6]. Heavy metals inhibit enzyme activity by binding with the amino acids in enzymes. The groups available for binding are amino, carboxyl and thiol (sulfhydryl) [7]. The enzyme urease is one in which enzyme activity can be inhibited by heavy metal ions [8]. Earlier studies of immobilized urease for the determination of urea have shown that the immobilized enzyme responded well to urea and was also stable for a long period of time [9,10]. Therefore, by measuring the decrease in activity of urease under the inhibitory effect of heavy metal ions it may be possible to create a sensor to detect them.

The enzyme urease catalyses the hydrolysis of urea according to:

$$H_2NCONH_2 + H_2O + 2H^+ \longrightarrow 2NH_4^+ + CO_2$$

$$CO_2 + H_2O \longrightarrow HCO_3^- + H^+$$

These reactions increase the abundance of charged products which then raise the conductivity of the solution; the effect can be detected by using conductivity electrodes [9,10]. In the presence of inhibitor(s) the activity of urease would decrease, and so would the conductivity. Thus it should be possible to detect this inhibition. The current work describes the use of a previously reported conductimetric enzyme sensor for urea [10] in a study of the inhibitory effects of heavy metal ions on immobilized urease, and the process of regeneration of the enzyme to provide an alternative method for heavy metal detection.

MATERIALS AND METHODS

Enzyme immobilization

Urease (urea aminohydrolase EC 3.5.1.5 from Jack Bean type IV 69 units/mg; Sigma, St. Louis, Missouri, USA) 10 mg/ml was added to wet glass beads (EKA Nobel AB, Surte, Sweden). The enzyme was immobilized on porous glass as described in an earlier work [10].

Instrumentation

The analytical system for urea using the conductivity electrodes was as in [10] (Fig.1). When the solution containing urea passes through the enzyme column, urea is degraded by urease into charged products, thus increasing the conductivity of the solution. The change is measured as the difference in conductivity compared to a baseline value obtained from a buffer solution without urea.

Chemicals

All chemicals used were of analytical grade. All solutions were prepared in 0.05 M glycine-NaOH buffer pH 8.8. The substrate solution was urea 10 mM. The regeneration solutions used were NaI and EDTA. The water used was nano pure to avoid metal contamination.

Procedure

Heavy metal ion inhibition

The buffer solution was pumped through the system with a peristaltic pump (Alitea C-4v, Ventur, Stockholm, Sweden) at a flow rate of 0.5 ml/min. The buffered solutions of urea and inhibitor were introduced as pulses in the continuous flow of buffer. A 0.25 ml pulse of 10 mM urea was pumped through the enzyme column. The charged products resulting from the hydrolysis of urea, catalysed by the immobilized urease, raised the conductivity of the solution. The effect was registered by the conductivity electrodes and recorded on a chart recorder (Fig.2). After the conductivity peak due to urea had been recorded, a 0.25 ml pulse of $CuSO_4$ solution was introduced, followed 3 minutes later by another 0.25 ml pulse of 10 mM urea. The degree of inhibition was expressed as

$$\% \text{ inhibition} = \frac{\text{conductivity peak reduction after inhibition}}{\text{conductivity peak before inhibition}} \times 100$$

The procedure was repeated for various concentration of $CuSO_4$ solution. Similar experiments were done using $HgCl_2$ with a 10 mM urea sample volume of 0.5 ml.

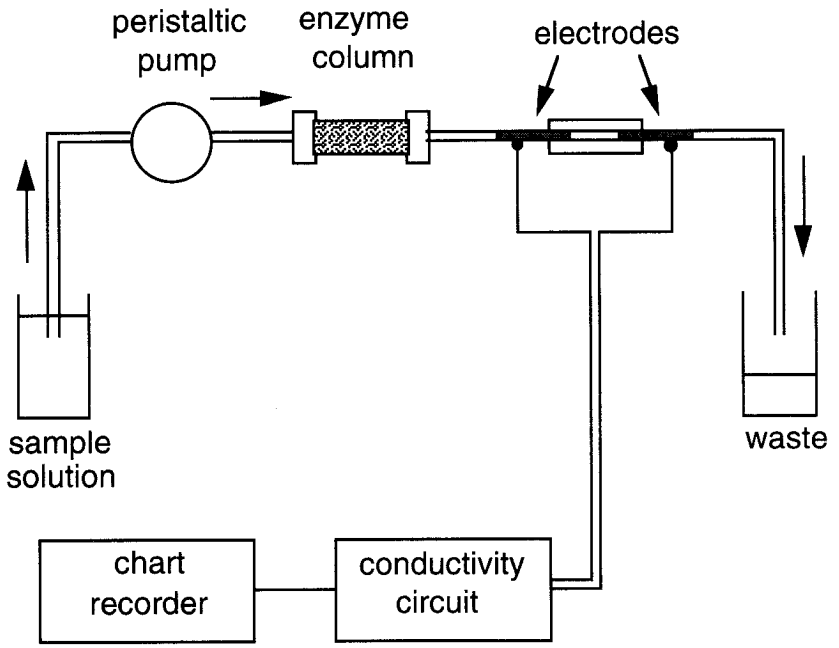

Fig.1 Schematic diagram showing the basic principle of the analytical system. Buffer, substrate or inhibitor is pumped through the column filled with immobilized urease. The solution then passes the conductivity electrodes where its conductivity is measured and recorded by a chart recorder.

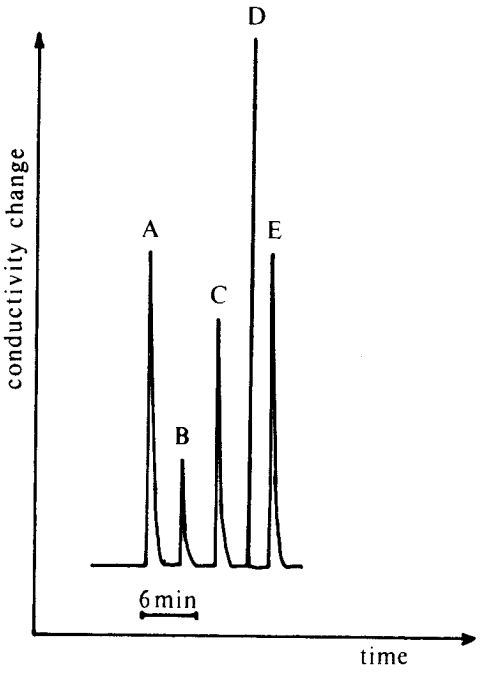

Fig.2 An analysis cycle of Cu^{2+} showing conductivity peaks when the solutions passed through the enzyme column. Solutions were introduced as pulses in a continuous flow of buffer, 0.5 ml/min. The cycle started with 0.25 ml of 10 mM urea (A) followed by 0.25 ml of the inhibitor, $CuSO_4$ solution (B). Another pulse of 0.25 ml 10 mM urea was then introduced (C). Regeneration of the enzyme column was done by passing 1.5 ml of 0.1 M NaI followed immediately by 1.5 ml of 0.01 M EDTA (D) (NOTE: the time scale was turned off here). After regeneration the response of the enzyme column to urea .was restored (E) and the system was ready for a new assay.

Enzyme regeneration

The enzyme column was regenerated by passing the following solutions to remove the heavy metal ions which bound to the immobilized enzyme

Cu^{2+}: 1.5 ml of 0.1 M NaI followed by 1.5 ml of 0.01 M EDTA

Hg^{2+}: 1.0 ml of 0.3 M NaI followed by 1.5 ml of 0.05 M EDTA

RESULTS AND DISCUSSION

Heavy metals determination

Copper(II) and mercury(II) ions were tested for their inhibitory effect on immobilized urease. Fig.2 shows the responses of the enzyme to urea before(A) and after (C) the introduction of inhibitor (B). Heavy metal ions passing through the enzyme column became bound to the enzyme molecules causing the enzyme activity to decrease, and thus lowering the conductivity peak. After regeneration by solutions of NaI and EDTA (D) the enzyme activity and the system were restored (E). Three assay cycles were done for each concentration of inhibitors and the relative decreases were calculated to give inhibition percentages which were then averaged. These averages were then plotted versus the heavy metal concentrations. The reproducibility of the degree of inhibition was satisfactory, the standard deviation for the percentage of inhibition in all cases was being less than ± 2. The inhibitory effects of copper(II) and mercury(II) are shown in Fig.3 and Fig.4 respectively; 0% denotes zero degree of inhibition in the absence of heavy metal ions. For both metals the percentage of inhibition is a linear function of the concentration. Copper was found to be less effective than mercury i.e. copper caused inhibition at concentrations above 10^{-4} M while mercury concentration showed an inhibitory effect above 10^{-6} M.

Effect of operation time and amount of enzyme on sensitivity

One of the problems associated with the use of immobilized enzyme is the denaturation of the enzyme over time. For substrate analysis where the immobilized enzyme is used for the determination of its substrate this problem is overcome by using a large access of enzyme. However, for the determination of inhibitors the amount of enzyme employed is generally quite small in order that the effect of the inhibitor can be seen; the inhibitory effect should be seen more clearly as the operation time of the enzyme column increases. This effect is shown in Fig.5: immediately after the preparation of immobilized enzyme (0 h) the degree of inhibition is rather small, since there are still plenty of active enzymes responding to the substrate. When the operation time increases, 100 h in this particular case, the denaturation of some of the enzymes reduces the amount of active enzyme, making the effect of inhibition more clearly apparent. The detection limit for copper(II) at 0 h is 0.3 mM and at 100 h is 0.1 mM and when used further, at 130 h of operation time, the limit was reduced to 0.05 mM. This indicates that by reducing the amount of active enzyme the inhibitory effect would increase.

However, it was found that using too little enzyme also has its disadvantages. A related series of experiments used a smaller amount of enzyme urease, by reducing the amount of enzyme per gram of carrier to one fifth of the previous preparation, i.e. only 2

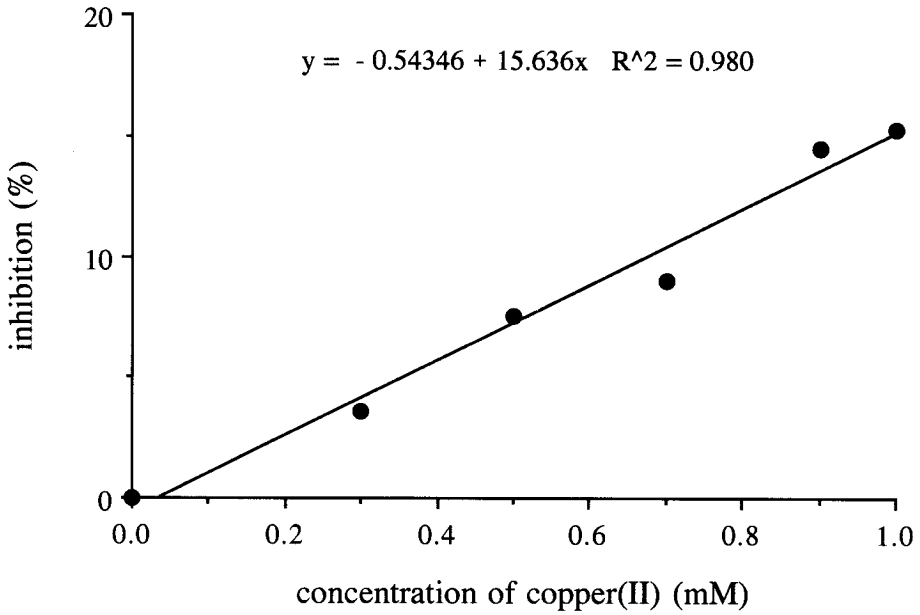

Fig.3 Percentage of inhibition of the enzyme column as a function of Cu^{2+} concentration.

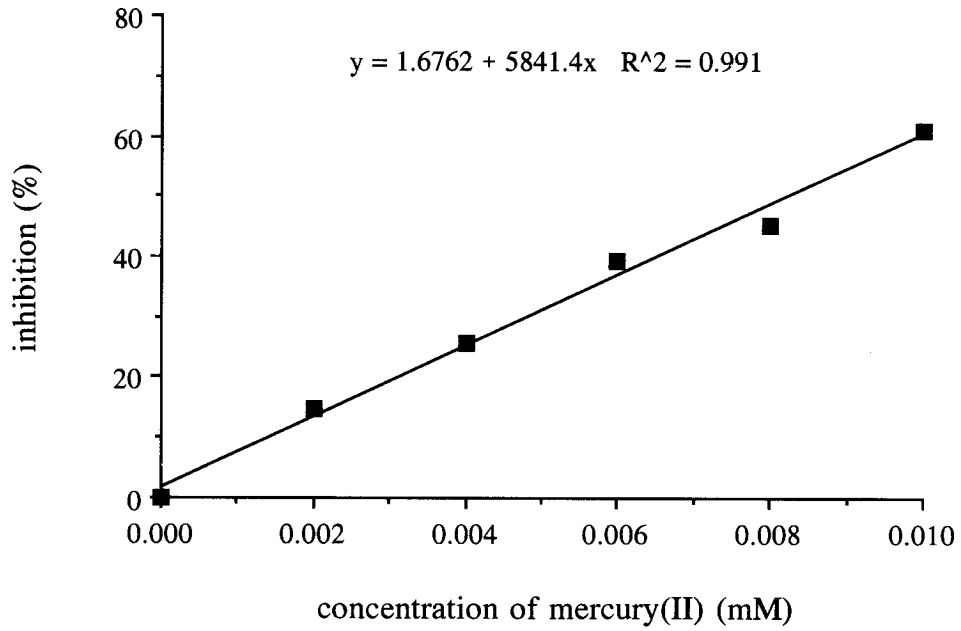

Fig.4 Percentage of inhibition of the enzyme column as a function of Hg^{2+} concentration.

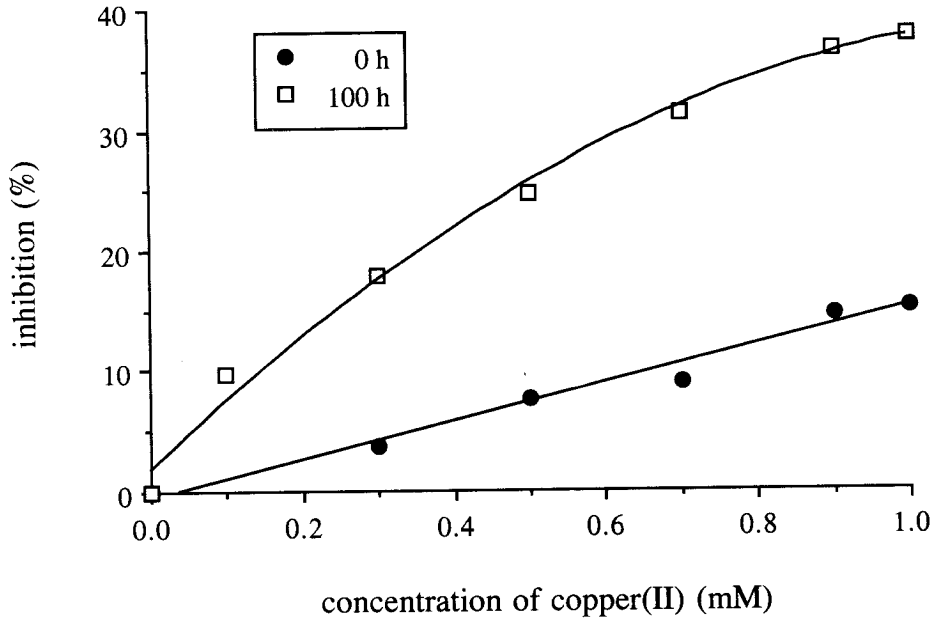

Fig.5 Percentage of inhibition of the enzyme column as a function of Cu^{2+} concentration at two different operation times of the enzyme column.

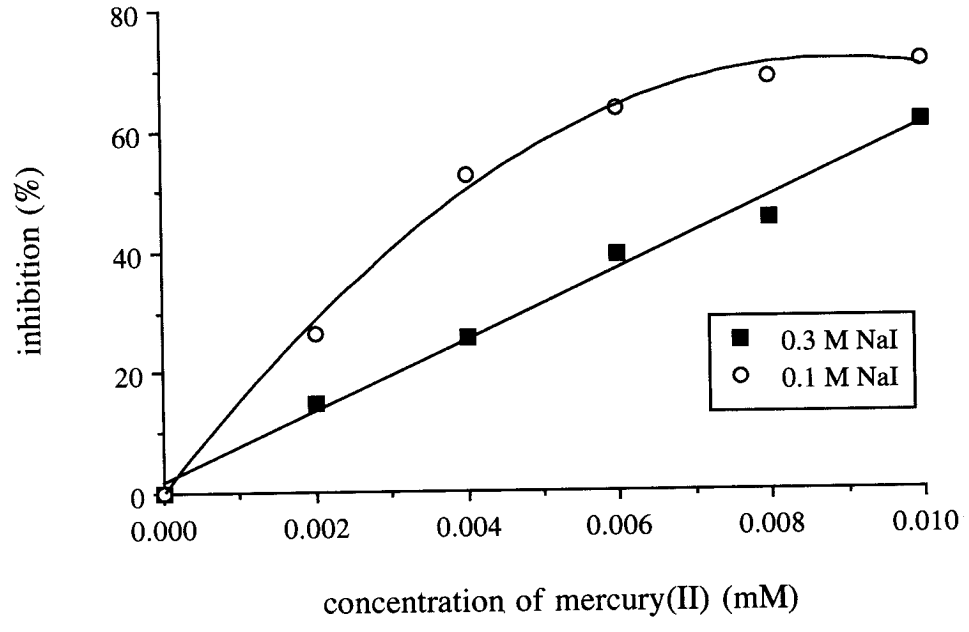

Fig.6 Percentage of inhibition of the enzyme column as a function of Hg^{2+} concentration, using two different concentrations of NaI solutions in the enzyme column regeneration process.

mg/ml of wet porous glass. In that study, the activity of the immobilized enzyme reduced too rapidly for the system to be useful. Further study of these variables is necessary.

From these results it may seem that the concentrations of heavy metals that can be detected with this system, 2 μM (0.4 ppm) for mercury and 0.1 mM (6 ppm) for copper, are still too high for environmental detection (i.e. for drinking water 0.001 ppm for mercury and 1.0 ppm for copper) reducing the amount of enzyme as mention above would help to increase the sensitivity of the system. Another way of increasing sensitivity is by varying the length of the inhibitor pulse. It was evident that the degree of inhibition depended on the amount of heavy metal ions present and not the sample size [5]. Thus, increasing the length of the inhibitor pulse may help to increase the detection limits of the system.

Enzyme regeneration

For the system to be useful it is necessary to regenerate the enzyme column for re-use. That is, the heavy metal ions have to be removed from the enzyme. In this study this was done by washing the column with solutions of NaI and EDTA. During the study of the optimum conditions for these solutions, the measurement of the conductivity of the solutions is very useful since their ions produce very high conductivity. Any remaining ions in the system could be seen clearly as a signal higher than the baseline level obtained with the low conductivity glycine buffer and the passing of the buffer could be continued until the remaining ions from NaI and EDTA were removed. Therefore, the suitable washing time could be found.

In earlier studies only solutions of NaI alone or EDTA alone were used to regenerate the enzyme. It was found that if NaI solution was used, response after the regeneration tended to be quite unstable, i.e. for the same concentration of urea the size of the response peaks tended to fluctuate. EDTA by itself could not remove all heavy metal ions particularly mercury(II). A mixture of NaI and EDTA was also tested. At high concentrations the enzyme regeneration was succesful, but it took quite a long time to wash the regenerating solution off the system. Lower concentrations helped to reduce the buffer washing time but could not completely remove the metal ions. The best method of regeneration was found to be washing the column with NaI solution followed immediately by EDTA. I^- from NaI forms soluble complexes with heavy metal ions and the solution of EDTA which followed helps to remove these heavy metal ions.

The optimum concentrations and volume of both NaI and EDTA were found to be 1.5 ml of 0.1 M NaI followed by 1.5 ml of 0.01 M EDTA for Cu^{2+} and 1.0 ml of 0.3 M NaI followed by 1.5 ml of 0.05 M EDTA for Hg^{2+}. Higher concentrations provide similar ability to remove the metals but cause the assay cycle to be longer, due to the longer time required for washing NaI and EDTA out of the system. Lower concentrations reduce the time, but may not completely remove the heavy metals. This is shown in an experiment using Hg^{2+} in which the concentration of NaI in the regeneration process was reduced to 0.1 M (Fig.6). The non linear correlation produced by the lower concentration of NaI suggested that there was insufficient amount of active enzyme for the determination of higher concentration of Hg^{2+}. The lower concentration of NaI, 0.1M, also gave a higher degree of inhibition than with 0.3 M NaI. This was probably caused by an accumulation effect of the heavy metals that had not been removed in the previous assay cycle.

CONCLUSIONS

This study shows that it is possible to detect heavy metal ions by their inhibitory effect on the enzyme urease using conductivity measurements. The enzyme system is restorable through regeneration by NaI and EDTA solutions. Although the sensitivity in this system is still too low for use in environmental measurements, further study to determine optimum conditions, such as longer pulses of inhibitors and/or smaller amounts of enzyme, may increase its sensitivity. Though urease can be inhibited by several heavy metal ions it may be possible to differentiate between the metal ions by stepwise regeneration of the enzyme column with different regenerating agent [11]. It may be argued that heavy metal ions can be determined rather conveniently by conventional analytical techniques such as atomic absorption spectroscopy, however, the development of an enzyme-based system may offer advantages such as continuous environmental monitoring and control. The system could be used to screen for the presence of heavy metals by measuring the inhibition. If the inhibitory effect exists then the species of these ions could be identify by other methods, e.g. atomic absorption spectroscopy.

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บทคัดย่อ

ผลการยับยั้งปฏิกิริยาของเอนไซม์ urease โดยโลหะหนักหาได้จากการเปรียบเทียบ conductivity peak ของสารละลายก่อนและหลังการผ่านสารยับยั้งเอนไซม์ conductivity peak นี้ เกิดจากสภาพการนำไฟฟ้าที่เพิ่มขึ้นเนื่องจากปฏิกิริยาไฮโดรไลซิสของยูเรียทำให้เกิดผลผลิตที่มีประจุ ความสัมพันธ์เชิงเส้นระหว่างเปอร์เซนต์การยับยั้งและความเข้มข้นของไอออนโลหะหนักจะอยู่ในช่วง 10^{-4}- 10^{-3} M สำหรับทองแดง (II) และ 10^{-6}- 10^{-5} M สำหรับปรอท (II) หลังจากการยับยั้งผ่านสารละลาย NaI ตามด้วย EDTA เพื่อให้คอลัมน์ของเอนไซม์สามารถทำงานได้ในสภาพเดิม