ENZYME THERMISTOR ANALYSIS OF HEAVY METAL IONS WITH USE OF IMMOBILIZED UREASE

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1. Introduction

The utilization of immobilized enzymes in clinical chemistry [1,2] and environmental analysis [3,4] is in rapid progress, but only a few reports are available on the determination of enzyme inhibitors [5,6]. Quantitative analysis of competitive inhibitors has been described in a few cases, viz., phosphate determination from the degree of inhibition of the alkaline phosphatase reaction [5]. The determination of non-competitive inhibitors has only been carried out in a semi-quantitative way, one example is the automated analysis of nerve gases using immobilized acetylcholine esterase [6].

Thermometric measurements based on immobilized enzymes has proved to be well suited for determination of compounds present in complex media [7–9], since the detection principle makes the system insensitive to variations in the physical properties (pH, ionic strength) of the solution [9].

This paper describes the use of the enzyme thermistor containing immobilized urease for quantitative and repeated determinations of the concentrations of various heavy metal ions in water solutions, in particular mercury and copper.

2. Materials and methods

The enzyme thermistor and accessory equipment for determination of serum urea were as in [10]. The enzyme thermistor column was filled with 0.5 ml immobilized urease preparation, coupled to controlled-pore glass beads as in [11]. Urease (EC 3.5.1.5, jack bean, type III, 28 units/mg; Sigma, St Louis, MO) was added, 38 mg/ml wet glass beads. In the determination of mercury 0.1 M sodium phosphate buffer, pH 7.0, was used, while 0.1 M sodium maleate buffer, pH 6.5, was used for determinations of copper. All chemicals were of analytical grade or better and special precautions were taken to avoid metal ion contaminations present in chemicals or in water used.

The enzyme thermistor was operated as in [10]. The degree of inhibition of the urease in the column was thus determined: a 30 s pulse of 0.5 M urea was pumped through the enzyme thermistor at 1 ml/min flow rate. After the corresponding temperature peak had been recorded, a 30 s sample pulse containing the inhibitor was introduced. Finally exactly 30 s after the sample pulse, another pulse of 0.5 M urea was introduced (fig.1). The degree of inhibition was thus expressed as the ratio of the temperature peaks obtained after, and before, introduction of the inhibitor. In order to obtain reproducible results the intervals between the different pulses were kept constant.

After inhibition with the metal ion the enzyme column was regenerated by passing the following solutions through the system for three minutes:
(i) Hg^{2+}; 0.3 M NaI + 50 mM EDTA.
(ii) Cu^{2+}; 0.1 M NaI + 50 mM EDTA.
The system was then ready for new determinations.

3. Results and discussion

A common problem associated with the use of immobilized enzymes over extended periods is the continuous, slow denaturation of the enzyme because

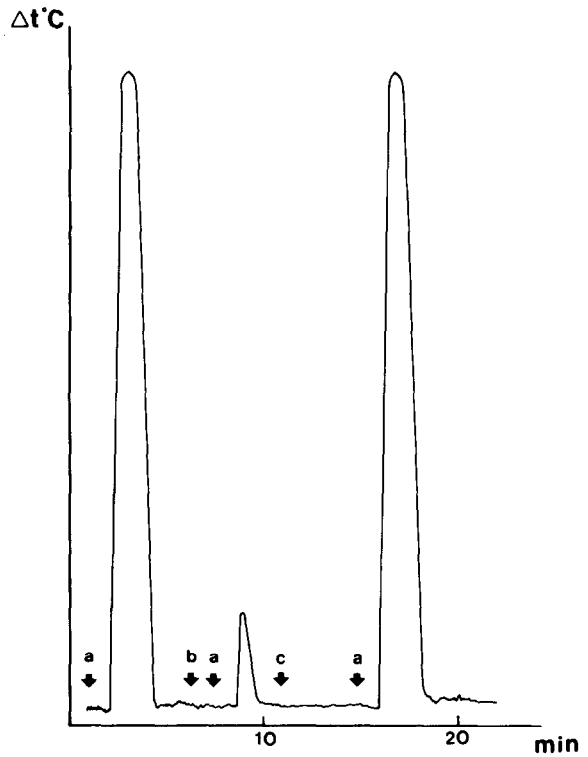

Fig.1. Schematic presentation of an assay cycle. The arrows indicate changes in the perfusion medium. Normally 0.1 M sodium phosphate buffer, pH 7.0, flow rate 1.0 ml/min is used. The cycle starts with assaying the activity of native urease by administration of a 30 s 0.5 M urea pulse (a). A 30 s inhibitor pulse (b) is administered and exactly after 30 s a new 0.5 M urea pulse (a) is introduced. Regeneration of the system is accomplished by a 3 min wash with iodide–EDTA solution (c). The system is then ready for another assay.

of inhibition by heavy metal ions [12]. This situation was utilized in the present study, which is based on the susceptibility of urease to inhibition by mercury and copper.

Preparations containing a large excess of enzyme is generally used for substrate analysis, whereas in the determination of inhibitors amounts of enzyme applied must be smaller [13]. It has also been shown that K_i app towards competitive inhibitors is changed due to the buffering capacity of the excess enzyme in the immobilized phase [14].

After thermal equilibration of the enzyme thermistor by continuously pumping of buffer through it, an assay scheme like that in fig.1 can be used. It is seen that inhibition of the urease is reflected in a lower response to the urea pulse following administration of the sample. With the high temperature response (approx. 1°C) obtained for a urea concentration of 0.5 M, a low amplification can be used resulting in a stable base line. The peak height can therefore be determined with a high degree of precision. A recorder with zero suppression will improve the range of detection of inhibition of the enzyme still more since, e.g., the upper 20% of the peak (0–20% inhibition) can be expanded over the whole chart.

The system was regenerated by washing of the column with a sodium iodide solution containing EDTA. Soluble HgI_4^{2-} complexes form according to the scheme:

$$E\text{–}Hg + 2I^- \longrightarrow E + HgI_2,$$

$$HgI_2 + 2I^- \longrightarrow HgI_4^{2-}$$

The tendency to complex formation is so strong that the enzyme activity is completely and repeatedly restored over many cycles even if the inhibition has been total [12]. Wide use of enzymic assay for non-competitive inhibitors has been prevented mainly by the requirement of methods for regenerating the enzyme activity, which can be accomplished either by reversible immobilization [15–17] or, as shown here, by a specific regenerating step. Figures 2 and 3 show standard curves, where 100% denotes urease activity in the absence of heavy metal ions. The shape of the standard curve is dependent upon the total activity of the immobilized enzyme preparation in relation to the concentration of the metal inhibitor. When working with large amounts of immobilized enzymes straight standard curves are obtained over a wide range of concentrations, whereas the use of smaller amounts results in non-linear relations at lower concentrations of inhibitors (fig.3).

Table 1 summarizes the concentrations of some metal ions leading to 50% inhibition of one particular immobilized urease preparation.

The useful concentration range for determination of the inhibitor can be selected by varying the amount of enzyme and length of the sample pulse. Thus, in one case the activity was decreased by 25% after introduction of a 5 min 10^{-9} M $HgCl_2$ pulse (0.2 ppb).

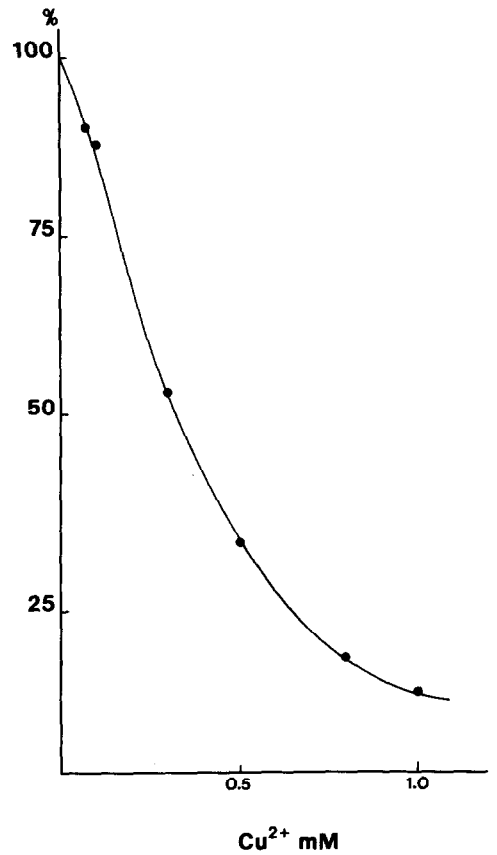

Fig.2. Standard curve for Cu^{2+} obtained in an experiment with high initial urease activity.

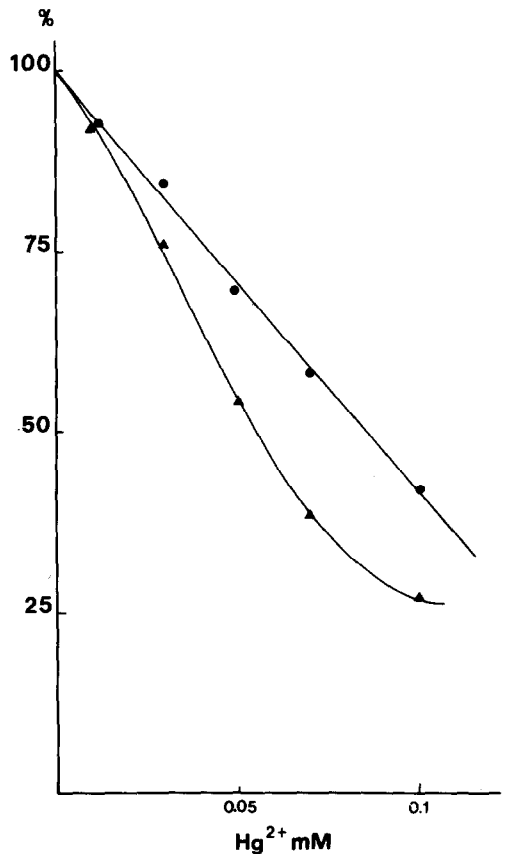

Fig.3. Standard curves for Hg^{2+} obtained with two different enzyme preparations; (▲) low substitution, (●) high substitution.

The procedure is at present available for discrete samples, but the system could presumably be relatively easily modified for continuous analysis as well.

If a crude solution contains more than one species of metal ions inhibiting the enzyme activity, it might be possible to differentiate between the metal ions by stepwise regeneration of the enzyme column with different regenerating agents. Alternatively, a gradient could be used. Both elution principles resemble those generally applied in the biospecific elution used in affinity chromatography.

Table 1
Heavy metal ion concentration required for 50% inhibition of a urease column with high enzymic activity

Metal ion	Concentration for 50% inhibition 0.5 min pulse
Hg^{2+}	50×10^{-6} M
Cu^{2+}	300×10^{-6} M
Ag^{+}	40×10^{-6} M

Thus, in preliminary experiments using mixtures of Hg^{2+} and Ag^{+} in imidazol buffer to inhibit the urease activity it was possible, after the initial assay of total degree of inhibition, to wash off selectively all Ag^{+} by a 6 min pulse of imidazol buffer before determination of the degree of inhibition by Hg^{2+}. The column was regenerated with the I^{-} wash described earlier.

Heavy metal ions can conveniently be assayed with the use of conventional analytical techniques, such as atomic absorption spectroscopy, but we feel

that the development of enzyme- or microorganism-based analytical systems of the type described here may offer advantages such as better registration of the effects of different pollutants on an eco-system and also allow the design of a system for continuous analysis. Such applications could include, e.g., waste water control. Techniques are also being developed for the assay of the equivalent to the value of biological oxygen demand (BOD) = measure of the total concentration of organic material in the water analyzed [18,19].

The use of immobilized enzymes in environmental control is still in its infancy. One instrument suitable for pesticide analysis combining immobilized butyryl choline esterase acting as the sensor with an electrochemical cell has been described [6]. In addition, some scientific reports are available on analysis of, e.g., CN^- [4], phenol [17] and other poisons such as uncouplers and arsenate [18].

In addition to allowing the quantitative determination of metal ions, the system described here should be particularly valuable for continuous environmental control since appearance of various heavy metal ions in a flow will quickly reduce an existing steady-state level of heat obtained on continuous passage of urea through the immobilized urease-containing enzyme thermistor unit. In this context it should be pointed out that after registration of a changed heat signal the nature of an inhibiting metal ion could be determined by, e.g., atomic sorption spectroscopy since it will remain bound to the column material.

Acknowledgement

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