IJBPAS, February, 2013, 2(2): 249-271

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

AN ULTRA-SENSITIVE ENZYME INHIBITORY METHOD FOR THE DETECTION OF MERCURY ION USING A NOVEL THIOL AMYLASE FROM THE PLANT *Tinospora cordifolia*

MUKHERJEE A, SENGUPTA S, JAJODIA M, HAIT S, PALIT S AND SENGUPTA S*

Department of Biotechnology, Heritage Institute of Technology, East Kolkata Township, Anandapur, Kolkata-700107, West Bengal, India. Tel: +91-33-24430445; Fax: 91-33-24430455

***Corresponding Author: E Mail: profs_sengupta@yahoo.ca**

ABSTRACT

A simple method is essentially required for routine analysis of Hg^{2+} in drinking water, assuring highest limit of 1 ppb in the same. We reported earlier the purification of a 43 KDa thiol amylase (containing 3 SH groups/ mole) from *Tinospora cordifolia*, capable of completely hydrolyzing both starch and amylopectin, without the formation of limit dextrin. We now report an analytical method based on the inhibition of thiol amylase activity by Hg^{2+}, monitored by starch-iodine color. Hg^{2+} showed an irreversible non-competitive inhibition with Ki = Ki' = 3.5 $\pm$ 0.25 nM and IC_{50} value of 1.9 $\pm$ 0.25 ppb. The method is much more sensitive than other enzymatic methods (thiol urease, bromelain, papain, trypsin) used for detection of Hg^{2+}. The method could detect Hg^{2+} at a minimum concentration of 0.5 ppb. The method was insensitive to the presence of (50 ppm) other heavy metals like Pb^{2+}, Cd^{2+}, and Cu^{2+} . The method remained insensitive upto 400 ppm of Ca^{2+}.

Keywords: Hg^{2+} Detection, Non-Competitive Inhibition, Thiol Amylase, Irreversible Inhibition, *Tinospora cordifolia*, Starch-Iodine Color

INTRODUCTION

Determination of mercury in drinking water is an essential part of environmental monitoring. Mercury is widely distributed in the earth's crust, sea, and ground and rain water. The toxic effects of mercury on biological systems through direct uptake as well as by the

accumulation in the system through food chains is well known **[1]**. Any possible pollution of mercury in drinking water requires rigorous assessments because of the steady intake of the pollutant on regular basis and its accumulation in the system. It has been estimated that approximately one-third of mercury circulating in the global environment is naturally occurring and approximately two-thirds released into the environment as a result of industrial and other human activities **[2]**. Li *et al.*, categorized the mercury contaminated sites in Asia into various types, based on different emission source categories, such as Hg pollution from Hg mining, gold mining, chemical industry, metal smelting, coal combustion, metropolitan cities, natural resources and agricultural sources **[3]**. Serious Hg pollution to the local environment were found in the area influenced by chemical industry, mercury mining and gold mining **[3]**. In the United States, both Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA) allow levels of total Hg^{2+} less than 0.002 milligram (mg)/L in both tap and bottled water **[4]**. The Bureau of Indian Standards (BIS) has laid down safety limits for drinking water at 0.001 mg of Hg^{2+}/L or 1 ppb **[5]**. A number of samples of groundwater in some industrial belts of India (Malkar industrial area, Maharashtra; Kandsar, Banarpal region of central Orissa; Panipat industrial state, Haryana etc.) have shown mercury contamination much higher (5- 268 ppb) than the recommended safe standards **[6]**. Detection of mercury in drinking water is likely to be a routine check-up for safety reasons, particularly in cities depending on centralized supply system. But ultra-trace mercury analysis in portable water, on regular basis is not possible at present as methods available require costly instruments and highly skilled operators. Large number of such ultra-sensitive instrumental methods have been reported with different limits of Hg^{2+} detection sensitivity (minimum detection limit varies from 0.05 ppb - 5 ppb), like fluorimetry, atomic absorption spectrometry [AAS] (cold vapor, electro thermal etc.), atomic fluorescence spectrometry [AFS], atomic emission spectrometry [AES], microwave induced plasma - atomic emission spectrometry [MIP-AES], X-ray fluorescence [XRF], etc **[7, 8]**. However, such sophisticated methodologies for environmental monitoring of mercury pollution are not expected to be available in every corner of the globe. In this context, it may be mentioned that numerous sensitive methods based on inhibition of enzyme activities (peroxidase, xanthine oxidase,

invertase, glucose oxidase, isocitric dehydrogenase, and urease) by Hg^{2+} have been reported for determination of Hg^{2+} in drinking water **[9, 10]**. Determination of heavy metal residues in water by inhibiting the activity of thiol proteases like papain **[11]**, bromelain **[12]**, and trypsin **[13]** have also been reported. However, the lowest detection limits of these methods are much above the recommended safety level of Hg^{2+} in drinking water.

We reported earlier that a non-cereal, non-leguminous plant *Tinospora cordifolia* Miers (Meninspermaceae family), produces substantial amount of extracellular saccharifying amylase in the plant body **[14]**. The enzyme was purified and was found to be a thiol amylase (43KDa) capable of hydrolyzing both soluble starch and amylopectin without the formation of any limit dextrin **[15]**. *T.cordifolia* is a climbing shrub, indigenous to and found widely distributed in India. It grows wild on unconditioned soil throughout the year and produces huge biomass in a short period. The plant is extensively used in folk and traditional medicine **[16]**. The present study reports the detailed inhibitory kinetics and mechanism of Hg^{2+} towards thiol amylase, and its use in determination of Hg^{2+} (in ppb level). The reaction involves complete dextrinization of soluble starch by thiol amylase and its inhibition by mercury ion which could be monitored by starch-iodine color reaction. The minimum detection limit of Hg^{2+} by the method was 0.5ppb which is 2 times lower than that allowed for drinking water. The method has already been patented by us **[17]**.

MATERIALS AND METHODS

Chemicals

Soluble starch (potato), dinitrosalicylic acid and Bradford reagent were purchased from sigma chemicals, USA. Mercuric chloride, iodine resublimed, potassium iodide, sodium acetate, calcium chloride, sodium thiosulphate were of analytical pure grade purchased from Merck, India.

Stems of *Tinospora cordifolia* Miers were collected at an altitude of 6.4 m near Heritage Institute of Technology campus, Anandapur, Kolkata, India in January 2009 by Dr. Abhishek Mukherjee. The plant was identified by Prof N.D. Paria, Department of Botany, University of Calcutta, India and a voucher specimen (CUH ACCN No. 635A) was deposited at the University of Calcutta, Herbarium.

Extraction of Enzyme

Healthy uninfected *Tinospora cordifolia* stems were collected. Enzyme was extracted according to the method mentioned in the

patent **[18]**. The process involved maceration of 800 g of washed small stem pieces of the plant (1 - 2 cm long) with 2 vol. (w/v) of 0.1 M acetate buffer pH 5.0 containing 10 mM cysteine (buffer A) at 50 °C followed by squeezing the paste through a nylon cloth. The greenish extract obtained (1400 mL) was treated with ethanol (15 % v/v) and the mixture kept overnight at 4 °C. The extract was filtered to remove a gummy precipitate settled overnight at the bottom. Enzyme protein was precipitated out from the filtrate at –10 °C by further addition of 4 vol. of chilled ethanol. The precipitate was collected by centrifugation at 4 °C and dissolved in buffer A and dialyzed against the same. This was used as a source of crude enzyme.

Purification of Enzyme

Purified thiol amylase was obtained from the crude extract by the method involving glycogen affinity precipitation, ammonium sulphate precipitation, gel-filtration (Biogel-P60) chromatography and HPGPLC as reported earlier by Mukherjee *et al.* **[15]**. The enzyme was homogeneous in SDS-PAGE.

Enzyme Assay

Amylase assay was determined using 1% (w/v) soluble starch as substrate in 0.1M acetate buffer pH 5.0 at 50 ^{0}C. Amount of reducing sugar formed was estimated by dinitrosalicylic acid reagent according to the method of Sengupta *et al*. **[19]**. 1 unit (U) enzyme activity was defined as the amount of enzyme that could produce 1 µmole of reducing sugar/min under the assay conditions. Protein was estimated using coomasie brilliant blue dye reagent (Bradford reagent) according to the guidelines given by the manufacturer **[20]**.

Preparation of Starch-Iodine Standard Curve and Determination of Dextrinizing Activity of Enzyme

0.1 N iodine solution containing 3 % (w/v) potassium iodide was prepared according to the method of Huggins and Russels **[21]**. The solution was standardized by titration with a standard sodium thiosulphate solution using starch indicator. The iodine solution was kept in a dark colored bottle and stored in a cool dark place. The stock iodine solution was diluted 10 times (0.01 N) with distilled water as and when required to be used as a color developing reagent. 0.1 % (w/v) starch stock solution was prepared. 0.1g of soluble starch was suspended in 100 mL of 0.1 M acetate buffer, pH 5.0 and well gelatinized by heating at 100 ^{0}C for 5 min with constant stirring.

Starch–iodine standard curve was prepared as follows: To the incubation mixtures (1mL) containing 0.1M acetate buffer, pH 5.0, different amount (5 - 200 µL) of stock starch solution was added to obtain a substrate

concentration range of 5 - 200 µg / mL. To this 40 µL of iodine reagent (0.01N) was added. Blue color developed was measured at 630 nm. The experiment was performed in triplicate sets and a standard curve (concentration vs OD) was plotted using the best fitting line.

Dextrinizing activity of the enzyme was determined as follows. The stock enzyme solution (100 U / mL) in buffer A was diluted 10 times with same buffer. Reaction mixtures (1mL), containing different concentration of enzyme (0.05 - 0.6 U) in 0.1M acetate buffer, pH 5.0 were kept at 50 $^{\circ}$C. Reaction was started by addition of 20 µL of 0.1 % (w/v) gelatinized starch solution. The tubes containing the reaction mixtures were taken out at fixed time intervals and the reaction was terminated by placing them in boiling water bath for 3 - 4 min. The tubes were then cooled to room temperature and 0.01 N iodine solution (40 µl) added to each. Optical density was measured at 630 nm using a UV-VIS Spectrophotometer (Shimadzu) and decolorization of starch-iodine color monitored. OD values obtained were expressed in terms of amount of starch (µg) dextrinized/min using the starch-iodine standard curve. The dextrinizing activity (DA) was calculated from the number of glycosidic linkages that are hydrolyzed / min under the assay condition according to the method of Sengupta *et al.* **[22]**. Five similar sets of experiments were carried out and data analyzed statistically using standard deviation.

Inhibition of Thiol Amylase by Hg^{2+}

Inhibitory effect of mercury ions on enzyme activity was determined as mentioned in the patent **[17]**. A stock solution (20 ppm) of Hg^{2+} (as mercuric chloride) was prepared in double distilled water. The reaction mixtures (1 mL) containing 0.2 U of enzyme (U calculated as mentioned above) in 0.1M acetate buffer, pH 5.0 was incubated with varying concentration (0.25 ppb - 1ppm) of Hg^{2+} for 10 min at room temperature. Reaction was started with addition of 50 µL of 0.1 % (w/v) starch solution. The reaction continued for 30 min at 50 $^{\circ}$C and was terminated by keeping the tubes in boiling water bath for 3-4 min. The tubes were cooled to room temperature and 0.01N iodine solution (40 µl) was added to each. Suitable 'control' tubes (containing enzyme and substrate but no Hg^{2+}) and 'blank' tubes (containing only starch and buffer) were also run parallel. The blue color developed in the tubes was measured at 630 nm (using a Shimadzu UV-VIS spectrophotometer) and thus a standard color index (indicating the amount of Hg^{2+}) was obtained. The amount of

inhibitor (Hg^{2+}) required to inhibit 50 % of enzyme activity was taken as IC_{50} value. Triplicate similar sets of experiments were carried out and data analyzed statistically using standard deviation. Similar set of experiment was also carried out using same amount (U) of ethanol precipitated dialyzed crude enzyme.

Inhibition Kinetics of Dextrinizing Activity of *T. cordifolia* Thiol Amylase by Hg^{2+} and Determination of its Inhibitory Constants

Inhibition kinetics of dextrinizing activity of *T. cordifolia* thiol amylase by Hg^{2+} was studied in detail by the **s**tarch-iodine decolorization method (the same above mentioned experimental protocol) using varying concentration (20 - 60 µg/mL) of gelatinized starch solution. Reaction mixtures (1mL), containing 0.2 U of enzyme in 0.1M acetate buffer, pH 5.0 was incubated with different concentration (0.25 ppb - 10 ppb) of Hg^{2+} for 10 min at room temperature. Reaction was started with addition of starch. The tubes containing the reaction mixtures were taken out at different time intervals and amount (µg) of starch dextrinized/ min was determined as mentioned earlier. Experiments were performed in five similar sets and standard deviation (SD) values calculated.

Enzyme inhibition constants were determined by the above mentioned assay protocol using 0.15 U/mL of enzyme, varying substrate (starch) concentration (20 - 60 µg/ mL) and 0.025 - 20 ppb of Hg^{2+}. Ki and Ki' values were determined using I/V vs I plot (Dixon pot) and S/V Vs I plot respectively using regression analysis.

Inhibitory Effect of Other Metal Ions on Thiol Enzyme Activity

Effect of other metal ions (Cd^{2+}, Pb^{2+}, As^{3+}, Ca^{2+}, Fe^{3+}) on enzyme activity was studied. A stock solution (2000 ppm) of different metal ions was prepared using their respective salts. The stock solutions were suitably diluted. Incubation mixtures (1mL) containing 0.2U of thiol amylase in 0.1M acetate buffer, pH 5.0 and different concentration (0.25 ppb – 1000 ppm) of various metal ions (Cd^{2+}, Pb^{2+}, $Cu^{2+}As^{3+}$, Ca^{2+}, Fe^{3+}) was kept at room temperature for 10 min. Reaction was started with addition of 50 µL of 0.1 % (w/v) gelatinized starch solution. Inhibition of enzyme activity by the metal ions was determined using iodine reagent as mentioned earlier. Triplicate similar sets of experiment were conducted and mean values calculated.

RESULTS

Dextrinization of Soluble Starch by Thiol Amylase Monitored by Starch-Iodine Color

Figure 1 shows the starch-iodine standard curve. With the protocol used, starch-iodine

color was linear till starch concentration of 60 µg/mL. Starch concentration below 20 µg/mL would give a very low OD value which was not suitable as control reading for determination of subsequent decolorization kinetics. The starch iodine color developed was stable till 12 h of study (data not shown).

Figure 2 shows the dextrinization of starch (20 µg/mL) by *T. cordifolia* amylase as monitored by starch-iodine color. It was observed that 0.1 - 0.4 U/mL of enzyme could dextrinize about 93 - 95 % of starch used and almost completely decolorize the blue color in about 30 min. 0.6 U/ml of enzyme did the same in about 20 min while 0.05 U/mL of enzyme took about 60 min for complete decolorization of starch-iodine color.

Inhibition Kinetics Study of Thiol Amylase by Hg^{2+}

Figure 3 [(a) - (c)] shows dextrinization kinetics and iodine decolorizaton kinetics of starch by *T.cordifolia* amylase in presence and absence of Hg. It was observed that 0.5 ppb of Hg^{2+} inhibited almost 11 - 14 % of enzyme activity within 30 min of incubation. Significant inhibition was observed in case of 1ppb Hg. The 'control' tubes containing no Hg^{2+} showed a sharp drop in OD value and became almost colorless by about 90 min. About 50 % of enzyme activity was inhibited within 15 min by 2ppb of Hg. 8ppb of Hg inhibited almost 90- 94 % of enzyme activity within 15 min of incubation. The figure clearly indicates that starch dextrinization reaches a plateau phase by about 60 – 90 min, no further significant drop in OD value is noticed in both 'control' and 'test' tubes. It was found that similar range of inhibition percentage was observed using the highest (80 µg/ mL) and lowest (20 µg/mL) substrate concentration. IC_{50} value was calculated to be in the range of 1.9 $\pm$ 0.25 ppb. Similar range of inhibition was also observed using same amount of ethanol precipitated dialyzed enzyme (data not shown).

Fig. 4 shows the standard color index (indicating the Hg^{2+} concentration in each tube) developed by residual un-digested starch in the tubes (due to inhibition of thiol amylase activity by Hg^{2+}) with iodine reagent. The 'control' tube was colorless and did not give a characteristic blue color with iodine reagent while the 'blank' tube gave a deep blue color. Fig. 4 also clearly indicates that the method ensures visual detection of Hg^{2+} minimum upto 0.5ppb or 0.0025 µM (2.5 nM). Hg^{2+} at a concentration of 10 ppb and higher gave similar intensity of blue color **(Figure. not shown)**.

Dixon plot (1/V vs I) and S/V Vs I plots showed the nature of inhibition to be non-competitive **(Figure 5)** with Ki = Ki' = 0.70 $\pm$

0.05 ppb (0.0035 ± 0.00025 µM or 3.5 ± 0.25 nM). **Table 1** shows a comparative study of some enzymatic methods for detection of Hg^{2+}. Urease from *Cucumis melo* (pumpkin) seeds used for detection of Hg^{2+} was inhibited by the heavy metal (Hg^{2+}) with Ki value of 1.26×10^{-1} µM, while the heavy metal (Hg^{2+}) inhibited *T.cordifolia* thiol amylase with Ki value of 0.0035 ± 0.00025 µM **(Table 1)**. Hg^{2+} inhibited crude and partially purified bromelain with an IC_{50} value of 0.65 - 0.8 µM and 0.45 - 0.575 µM respectively. Minimum detection limit of Hg^{2+} were found to be 1 and 500 ppb using acid urease and glucose oxidase respectively, while the detection limit was found to be 0.5ppb using *T.cordifolia* thiol amylase **(Table 1)**.

Effect of Various Metal Ions on Activity of Thiol Amylase

Figure 6 shows the effect of other metal ions on enzyme activity and on the present methodology. Thiol amylase activity was not inhibited by the presence of metal ions like As^{3+}, Pb^{2+}, Cd^{2+}, Cu^{2+}, Fe^{3+}, Ca^{2+} upto a concentration of 50 ppm. Cd^{2+} at 75 ppm inhibited about 20- 22% of enzyme activity, while Cu^{2+} at the same concentration inhibited about 15-18 % of enzyme activity. Ca^{2+} showed no inhibition upto 400 ppm. It could inhibit only about 10-20 % of enzyme activity at a very high concentration of 800-1000 ppm, while Cu^{2+} at similar concentration inhibited about 65-70 % of enzyme activity. Pb^{2+} inhibited enzyme activity beyond 100 ppm concentration. As^{3+} and Fe^{3+} showed very little inhibition (about 10 %) at 800-1000 ppm.

DISCUSSION

A 43 KDa thiol amylase (specific activity = 546.2 U/mg of protein) was purified from *Tinospora cordifolia* by glycogen precipitation, ammonium sulfate precipitation, gel filtration chromatography, and HPGPLC **[15]**. The enzyme was optimally active in pH 6.0 and was stable at a pH range of 3.5-7.5. Thiol blockers like p-chloromercuric benzoate (PCMB), iodoacetic acid, iodoacetamide, dithionitrobenzoic acid (DTNB) inhibited enzyme activity. Inhibition of enzyme activity by DTNB was nullified by addition of cysteine to the assay system. It was found that 3 SH groups were present/ mole of enzyme **[15]**.

The starch–iodine standard curve experiment showed the intensity of the blue color developed was proportional and linear upto a concentration of 60 µg/mL of starch **(Figure 1)**. The standard curve clearly indicated that substrate (starch) concentration range of 20 - 60 µg/ mL was most suitable for the dextrinization assay using iodine reagent. Starch concentration below 20 µg/mL gave a

very low OD value and therefore could not be considered suitable for dextrinization experiments.

Starch dextrinization assay using thiol amylase from *T. cordifolia* indicated that an enzyme concentration range of 0.1- 0.4 U/mL and substrate concentration of 20-60 µg/mL was ideal for detection of Hg^{2+} ions within a suitable reaction time (30 – 60 min). An enzyme concentration range of 0.1 – 0.4 U/ mL could completely dextrinize starch within 60 min and hence no blue color developed in the 'control' tubes **(Figure 4)**. It may be mentioned that fungal amylase (*A. oryzae*) acts only on α-1,4 linkages of starch, bypassing the α-1,6 glycosidic linkages and therefore could not directly decolorize the starch-iodine blue color, but changed the color into red as limit dextrin remained undigested in the reaction mixture. However, *T. cordifolia* thiol amylase hydrolyzed both amylopectin and starch equally thus acting on both α-1,4 and 1,6 glycosidic linkages **[14]**. The enzyme could even hydrolyze glycogen much more efficiently than *A. oryzae* amylase **[14]**.

T. cordifolia thiol amylase was found to be supersensitive to Hg^{2+} which could inhibit 50 % of enzyme activity at a concentration of 1.9 $\pm$ 0.25 ppb. Enzyme inhibition kinetics experiments showed that similar amount of inhibition % was observed using the maximum and minimum concentration of starch, thus indicating the inhibition was not dependent upon the substrate concentration **(Figure 3 and Figure 5)**. Almost Inhibition experiments showed (considering the OD values at 630 nm) minimum detection limit of Hg^{2+} was 0.5ppb (0.0025 µM or 2.5 nM) **(Figure 4)**. It must be mentioned here that according to a WHO report (23), the minimum limit of detection of Hg^{2+} in water is 0.6 ppb using inductively coupled plasma (ICP), and 5 ppb by flame atomic absorption spectroscopy. The present method could detect Hg^{2+} in the range of 0.5 ppb to 10 ppb (Fig. 4). 10ppb of Hg^{2+} almost completely inhibited enzyme activity **(Figure 3)**; therefore a higher Hg^{2+} concentration would give the same intensity of blue color. However, Hg contamination above 10 ppb can be measured easily by suitably diluting the contaminated water sample. The method worked equally well using crude ethanol precipitated dialyzed enzyme. The inhibition time-kinetics experiments **(Figure 3)** showed that Hg^{2+} inhibited thiol amylase in irreversible manner as even after 12 h of incubation with enzyme, the 'test' tubes containing Hg^{2+} gave a positive test (blue color) with iodine solution, indicating the presence of residual starch. Thus the blue

color once developed remains stable for a long time thus facilitating the process of Hg^{2+} detection. The increase of substrate concentration did not lift up the inhibitory effect of Hg^{2+} **(Figure 3)**. The kinetics study, using Dixon plot and S/V vs I plot, revealed that Hg^{2+} inhibited thiol amylase in a non-competitive manner, with Ki=Ki' = 0.0035 $\pm$ 0.00025 µM **(Figure 5)**.

There are several reports on enzyme-inhibitory methods for the determination of Hg^{2+} and other heavy metals. Recently, a thiol urease from *Cucumis melo* (pumpkin) seeds was used for determination of Hg^{2+} **[24]**, the IC_{50} and Ki values were 1 and 0.126 µM respectively **(Table 1)**, thus being much more less sensitive than *T.cordifolia* thiol amylase method (IC_{50} and Ki = 0.010 $\pm$ 0.001 µM and 0.0035 $\pm$ 0.00025 µM respectively). Moreover, the major drawback of using urease is that the method suffers from high interference from ammonia in the environment **[25]**. Free and alginate immobilized urease from *Cucumis melo* (pumpkin) seeds was also used for detection of Cd^{2+} with a Ki = 1.41 X 10^{-5} M **[26]**. It is known that thiol enzymes are sensitive towards heavy metals especially Hg as it binds to the sulphydryl group (s) of enzymes thereby inactivating them **[25]**. Papain, a thiol protease, is also sensitive towards Hg and several studies on bioremediation of Hg using papain have been reported **[27]**. Shukor *et al.* **[11]** reported a heavy metal detection method using papain with casein-coomassie-dye-binding assay. The method however, showed a much less sensitive IC_{50} value of 1.95 µM **(Table 1)**, about 100 times less sensitive than *T.cordifolia* thiol amylase. Similar Hg^{2+} determination assay test was developed using both crude and partially purified bromelain and also trypsin. However, these methods too were much less potent (about 50 - 60 times less sensitive) than *T.cordifolia* thiol amylase **(Table 1)**.

An amperometric biosensor developed for the indirect determination of Hg^{2+} by inhibition of glucose oxidase immobilized on a carbon paste electrode bulk modified with manganese dioxide **[28]**, is almost 1000 times less potent that *T.cordifolia* thiol, amylase method, on the basis of minimum detection limit **(Table 1)**. Several other biosensors were also developed using enzyme inhibitive methodology for the detection of Hg^{2+} in water eg. invertase-glucose oxidase based biosensor with a detection range 2-10 ppb **[29]**, acid urease based optical sensor system, with a minimum detection limit of 1ppb **[30]**, methylene blue-mediated enzyme biosensor using horseradish peroxidase with a minimum detection limit of was 0.1ppb **[31]**, lactate dehyrogenase based

biosensor with an IC_{50} value of 0.013 µM **[32]**. A biologically active polymer created by co-polymerisation of glutaraldehyde with enzyme pyroloquinoline quinone dependent glucose dehydrogenase from *Gluconobacter* sp. 34 was developed for detection of heavy metals like Cd^{2+}, Pb^{2+} minimum at a concentration of 0.15mM **[33]**. It must be mentioned here that *T. cordifolia* thiol amylase was stable at room temperature as a lyophilized powder during 18 months of study and was stable for about 6 months as an aqueous extract at 4°C **[14]**. The enzyme showed optimal activity at a temperature of 60°C and remained stable upto 55°C, thus making it excellent for outdoor analysis.

Figure 6 shows that the present ultra-sensitive method of detection of Hg ion in water can work efficiently in presence of other metal ions contamination (upto 50 ppm). Heavy metals like Pb, Cu, and Cd, below 50 ppm, did not inhibit the activity of thiol amylase. This safety range is quite high and heavy metal contamination above 50 ppm is rare. The thiol enzyme was not affected by presence of Ca^{2+} (upto a concentration as high as 400 ppm) thus making it very suitable for analysis of different types of water sample. The enzyme remained insensitive to the presence of Fe^{3+} (upto 200 ppm). The enzyme was practically insensitive to the presence of As^{3+}, inhibiting only about 10 % of enzyme activity at a high concentration of 800-1000 ppm. Insensitivity of thiol amylase towards arsenic is very beneficial for its use as an analytical kit especially in a country like India, where there are several reports on As^{3+} contamination, especially in eastern part of the country.

CONCLUSION

The enzyme-inhibitory method described above for the detection of Hg^{2+} in drinking water appears to be very sensitive, much more potent than the much discussed thiol urease (*Cucumis melo*), bromelain, papain and glucose–oxidase methods reported recently **(Table 1)**. Hg^{2+} inhibits thiol amylase in an irreversible, non-competitive manner, thus the inhibition is lifted up neither by the increase in substrate concentration nor with increase in time. The method is very simple, visually detectable and does not require the use of any costly or purified enzyme, nor any costly reagents. The test can be done even in remote place, without any sophisticated instrumental support for assessing possible Hg^{2+} contamination. The method can easily be developed into a simple kit.

REFERENCE

[1] Bradstreet J, David G, Kartzinel J, Adams J and Geier M, A Case-Control Study of Mercury Burden in Children

with Autistic Spectrum Disorders, J. Am. Physicians and Surgeons, 8(3), 2003, 76-79.

[2] Spadaro JV and Rabl A, Global Health Impact and Costs due to Mercury Emissions, Risk Analysis, 28 (3), 2008, 603-613.

[3] Li P, Feng XB, Qiu GL, Shang LH and Li ZG, Mercury Pollution in Asia: A Review of the Contaminated Sites, J. Hazardous Materials, 168 (2-3), 2009, 591-601.

[4] Ware GW, Mercury, USEPA office of drinking water health advisories, Review of Environ. Contamination and Toxicol., 107, 1989, 93-102.

[5] Deshpande K, Mishra RK and Bhand S, A High Sensitivity Micro Format Chemiluminescence Enzyme Inhibition Assay for Determination of Hg (II), Sensors, 10 (7), 2010, 6377-6394.

[6] Status of groundwater quality in India -part – I, Central pollution control board (Ministry of environment and forests), 2007, Groundwater quality series: gwqs/ 09/2006-2007.

[7] Morita M, Yishinaga J and Edmonds JS, The Determination of Mercury Species in Environmental and Biological Samples (Technical Report), Pure and Applied Chem., 70 (8), 1998, 1585-1615.

[8] Clevenger W, Smith BW and Winefordner JD, Trace Determination of Mercury: A Review, Critical Reviews in Analytical Chem., 27 (1), 1977, 1-26.

[9] Krawczyn´ski T, Moszczyn´ska MG and Trojanowicz M, Inhibitive Determination of Mercury and Other Metal Ions by Potentiometric Urea Biosensor, Biosensors and Bioelectronics,15 (11-12), 2000, 681-691.

[10] Jung K, Bitton G and Koopman B, Assessment of Urease Inhibition Assays for Measuring Toxicity of Environmental Samples, Water Res., 29 (8), 1955, 1929-1933.

[11] Shukor MY, Baharom NA, Abd. Rahman F, Abdullah MP, Shamaan NA and Syed NA, Development of a Heavy Metals Enzymatic-Based Assay Using Papain, Analytica Chimica Acta, 566 (2), 2006, 283-289.

[12] Shukor MY, Baharom NA, Abd. Rahman F, Abdullah MP, Shamaan NA and Syed NA, An Inhibitive Determination Method for Heavy

Metals Using Bromelain, a Cysteine Protease, Applied Biochem. and Biotechnol., 144 (3), 2008, 283-291.

[13] Shukor MY, Baharom NA, Abd. Rahman F, Abdullah MP, Shamaan NA and Syed NA, The Development of an Inhibitive Determination Method for Zinc Using a Serine Protease, J. Environ. Biol., 30 (1), 2009, 17-22.

[14] Mukherjee A, Sengupta S, Ray L and Sengupta S, Evaluation of *Tinospora cordifolia* Amylase as a Commercial Digestive Enzyme of Plant Origin, J. Herbs Spices and Medicinal Plants, 18, 2012, 58-76.

[15] Mukherjee A, Ghosh AK and Sengupta S, Purification and Characterization of a Thiol Amylase over Produced by a Non-Cereal Non-Leguminous Plant, *Tinospora cordifolia*, Carbohydrate Res., 345, 2010, 2731-2735.

[16] Singh SS, Pandey SC, Srivastava S, Gupta VS, Patro B and Ghosh AC, Cheimstry and Medicinal Properties of *Tinospora cordifolia* (Gudichi), Indian J. Pharmacol., 35, 2003, 83-91.

[17] Sengupta S, Mukherjee A and Sengupta S, A Process for the Development of an Ultra Sensitive Test Method for Detection of Mercury Ion, Indian Patent Application No. 585/KOL/2011, 2011.

[18] Sengupta S, Mukherjee A and Sengupta S, A Process for the Preparation of a Complete Enzyme Composition from the Plant *Tinospora Cordifolia* Useful for Hydrolysis of Starch into Glucose as the Major End Product, Indian Patent Application No. 1989/KOL/2008, 2008.

[19] Sengupta S, Jana M, Sengupta D, Naskar AK, A Note on the Estimation of Microbial Glycosidase Activities by Dinitrosalicylic Acid Reagent, Applied Microbiol. and Biotechnol., 53 (6), 2000,732-735.

[20] Bradford M, A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein Dye Binding, Analytical Biochem., 72, 1976, 248-254.

[21] Huggins C and Russell PS, Colorimetric Determination of Amylase, Annals of Surgery, 128 (4), 1948, 668-678.

[22] Sengupta S and Sengupta S, Regulation by Amino Acids of α-Amylase and Endo-B(1→4)-Glucanase Induction in Mycelial Culture of the Mushroom *Termitomyces clypeatus*, Canadian J. Microbiol., 36 (9), 1990, 617-624.

[23] Mercury in drinking-water. Background document for development of WHO Guidelines for Drinking-water Quality, 1995, WHO/SDE/WSH/05.08/10.

[24] Prakash O, Talat M, Hasan SH and Pandey RK, Enzymatic Detection of Mercuric Ions in Ground-Water from Vegetable Wastes by Immobilizing Pumpkin (*Cucumis melo*) Urease in Calcium Alginate Beads, Bioresource Technol., 99 (10), 2008, 4524-4528.

[25] Masdor NA, Said NAM, Partial Purification of Crude Stem Bromelain Improves its Sensitivity as a Protease Inhibitive Assay for Heavy Metals, Australian J. Basic and Applied Sci., 5 (10), 2011, 1295-1298.

[26] Prakash O, Talat M, Hasan SH and Pandey RK, Factorial Design for the Optimization of Enzymatic Detection of Cadmium in Aqueous Solution Using Immobilized Urease from Vegetable Waste, Bioresource Technol., 99 (16), 2008, 7565-7572.

[27] Bhattacharyya A, Dutta S, De P, and Basu S, Removal of Mercury (II) from Aqueous Solution Using Papain Immobilized on Alginate Bead: Optimization of Immobilization Condition and Modeling of Removal Study, Bioresource Technol., 101 (24), 2010, 9421-9428.

[28] Samphao A, Rerkchai H, Jitcharoen J, Nacapricha J and Kalcher K, Indirect Determination of Mercury by Inhibition of Glucose Oxidase Immobilized on a Carbon Paste Electrode, International J. Electrochemical Sci., 7(2012), 2002, 1001-1010.

[29] Amine A, Cremisini C and Palleschi G, Detection of Mercury Compound Using Invertase-Glucose Oxidase-Based Biosensor, Proc. SPIE, 2504, 1995, 209-220.

[30] Preininger C, The Enzymatic Determination of Mercury and Copper Using Acid Urease, The Effects of Buffers, Microchimica Acta, 130 (3), 1999, 209-214.

[31] Han S, Zhu M, Yuan Z, Li X, A Methylene Blue-Mediated Enzyme Electrode for The Determination of

Trace Mercury (II), Mercury (I), Methylmercury, and Mercury–Glutathione Complex. Biosensors and Bioelectronics, 16 (1-2), 2001, 9-16.

[32] Tan I, Erhan E, Karagoz P and Ozkan M, Determination of Mercury and Nickel by Amperometric Biosensor Prepared with Thermostable Lactate Dehydrogenase, Transactions of Nonferrous Metals Society of China, 21 (10), 2011, 2332-2338.

[33] Lapenaite I, Kurtinaitienė B, Pliuškys L, Laurinavičius V, Bachmatova I, Marcinkeviciene L and Ramanavicius A, Application of PQQ-GDH based polymeric layers in design of biosensors for detection of heavy metals, Materials Sci., 9(4), 2003, 431-435.

LEGENDS TO FIGURES AND TABLES

Figure 1: Standard Curve for Starch-Iodine Color

To the incubation mixtures (1mL) containing 0.1M acetate buffer, pH 5.0, different amount (5-200 µL) of stock starch solution (0.1 %; w/v) was added to obtain a substrate concentration range of 5-200 µg/mL. To this 40 µL of iodine reagent (0.01N) was added. Blue color developed was measured at 630 nm. The data points are average values for triplicate sets of similar experiments.

Figure 2: Iodometric Determination of *T. Cordifolia* Thiol Amylase Activity

Reaction mixtures (1mL), containing different concentration of enzyme (0.05 - 0.6 U) in 0.1M acetate buffer, pH 5.0 were kept at 50 ^{0}C. Reaction was started by addition of 20 µL of 0.1 % (w/v) gelatinized starch solution. Dextrinization activity was monitored as mentioned in the text. The data points are average values for triplicate similar sets of experiments and error bars represent sample standard deviation.

0.6 U/mL, 0.4 U/mL, 0.3 U/mL, 0.2 U/mL, 0.1 U/mL, 0.05 U/mL

Figure 3: Effect of Hg2+ on Thiol Amylase Activity And Starch-Iodine Decolorisation Kinetics

Reaction mixtures (1mL), containing 0.2 U of enzyme in 0.1M acetate buffer, pH 5.0 was incubated with different concentration (0.25 ppb- 10 ppb) of Hg^{2+} for 10 min at room temperature. Reaction was started with addition of starch. Decolorization kinetics was

monitored as mentioned in the text. The data represent the average of five similar sets of experiments and error bars represent sample standard deviation.

Control, 0.25pp, 0.5 ppb, 1ppb, 2ppb, 4ppb, 8ppb, 10ppb

Figure 4: The Standard Color Index for Different Concentration (0.5 Ppb -10 Ppb) of Hg^{2+}

The reaction mixtures (1 mL) containing 0.2 U of enzyme in 0.1M acetate buffer, pH 5.0 was incubated with varying concentration (0.25 ppb - 1ppm) of Hg^{2+} for 10 min at room temperature. Reaction was started with addition of 50 µL of 0.1 % (w/v) starch solution. The reaction continued for 60 min at 50 ^{0}C and was terminated by keeping the tubes in boiling water bath for 3-4 min. The tubes were cooled to room temperature and 0.01N iodine solution (40 µl) was added to each. The blue color developed in the tubes was measured at 630 nm. The Blank tube contains only buffer and substrate, while the 'Control' tube contains only enzyme (in buffer) and substrate.

Figure 5: Determination of Inhibitory Constants of Hg^{2+}

Inhibition kinetics was determined as mentioned in the text using 0.15 U/mL of enzyme, varying substrate concentration (20 - 60 µg/mL) and 0.025- 20 ppb of Hg^{2+}. Ki and Ki' values were determined using I/V vs I plot (Dixon pot) and S/V vs I plot respectively. The data points are average values for triplicate sets of similar experiments.

Figure 6: Effect of Other Metal Ions on Thiol Amylase

Reaction mixtures (1mL) containing 0.2U of thiol amylase in 0.1M acetate buffer and different concentration (0.25 ppb – 1000 ppm) of various metal ions (Cd, Pb, As, Ca, Fe^{3+}) was kept at room temperature for 10 min. Reaction was started with addition of starch. Dextrinizing activity was determined as mentioned in the text.

Fe^{3+}, As^{3+}, Ca^{2+}, Pb^{2+}, Cd^{2+}, Cu^{2+}

Table 1: Comparison of Different Enzymatic Methods for Detection of Hg2+

Table 1: Comparison of Different Enzymatic Methods for Detection of Hg^{2+}

Source enzyme	Method	IC_{50}	Minimum detection limit	Inhibitory constant (Ki)
Urease from pumpkim (*Cucumis melo*)[24]	Immobilization in calcium alginate beads	1µM	-	0.126 µM (non-competitive)
Crude bromelain[12]	Casein-coomassie-dye-binding assay	0.13 - 0.16 ppm (0.65 - 0.8 µM)	-	-
Partially purified bromelain [25]	Casein-coomassie-dye-binding assay	0.09 - 0.115 ppm (0.45 - 0.575 µM)	--	-
Papain [11]	Casein-coomassie-dye-binding assay	0.39ppm (1.95 µM)	-	--
Trypsin[13]	Casein-coomassie-dye-binding assay	16.38 ppm (81.9 µM)	1.35ppm (6.75 µM)	-
Acid urease [30]	Coupled to an optical sensor system	-	1ppb (0.005 µM)	-
Glucose oxidase from *Aspergillus niger*[28]	Immobilized on a carbon paste electrode bulk modified with MnO_2	-	0.5ppm (2.5 µM)	-
Thiol amylase from *T.cordifolia*	Starch-iodine color reaction	1.9 $\pm$ 0.25 ppb (0.010 $\pm$ 0.001 µM)	0.5ppb (0.0025 µM)	0.70 $\pm$ 0.5 ppb (0.0035 $\pm$ 0.00025 µM) (Non-competitive)

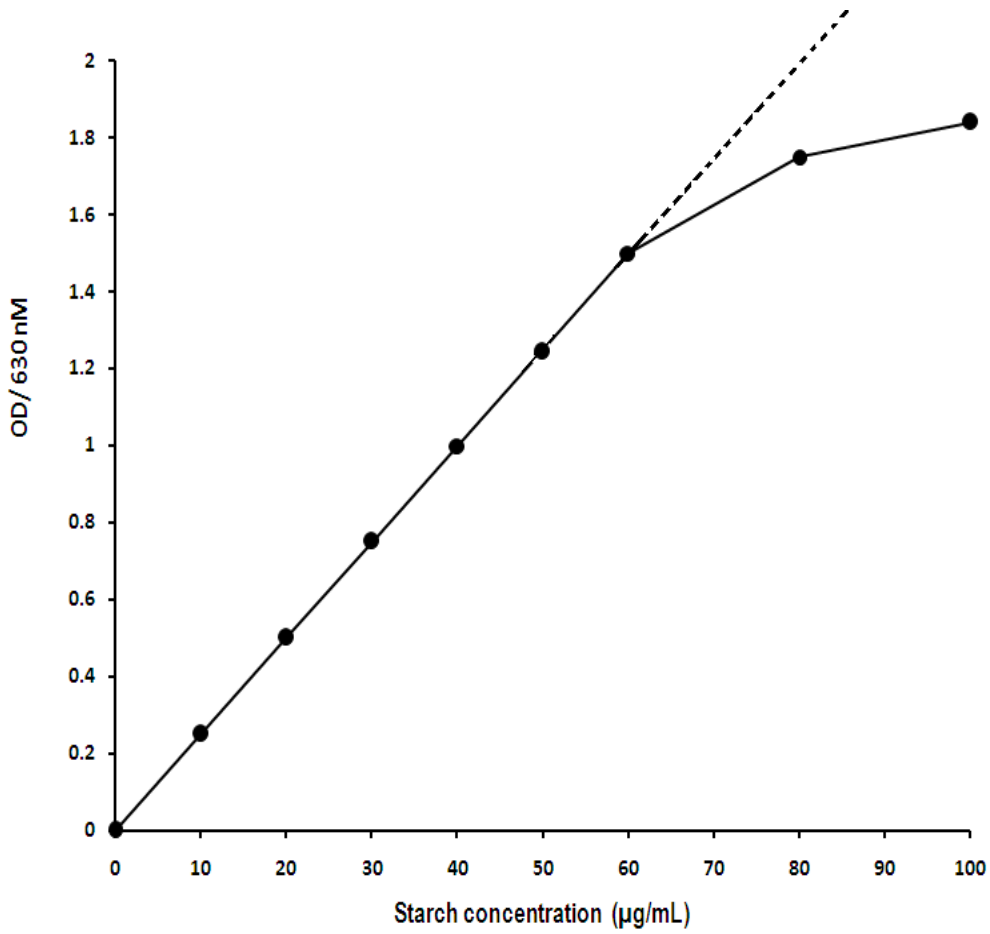

OD/630nM
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
80
90
100
Starch concentration (µg/mL)

Figure 1

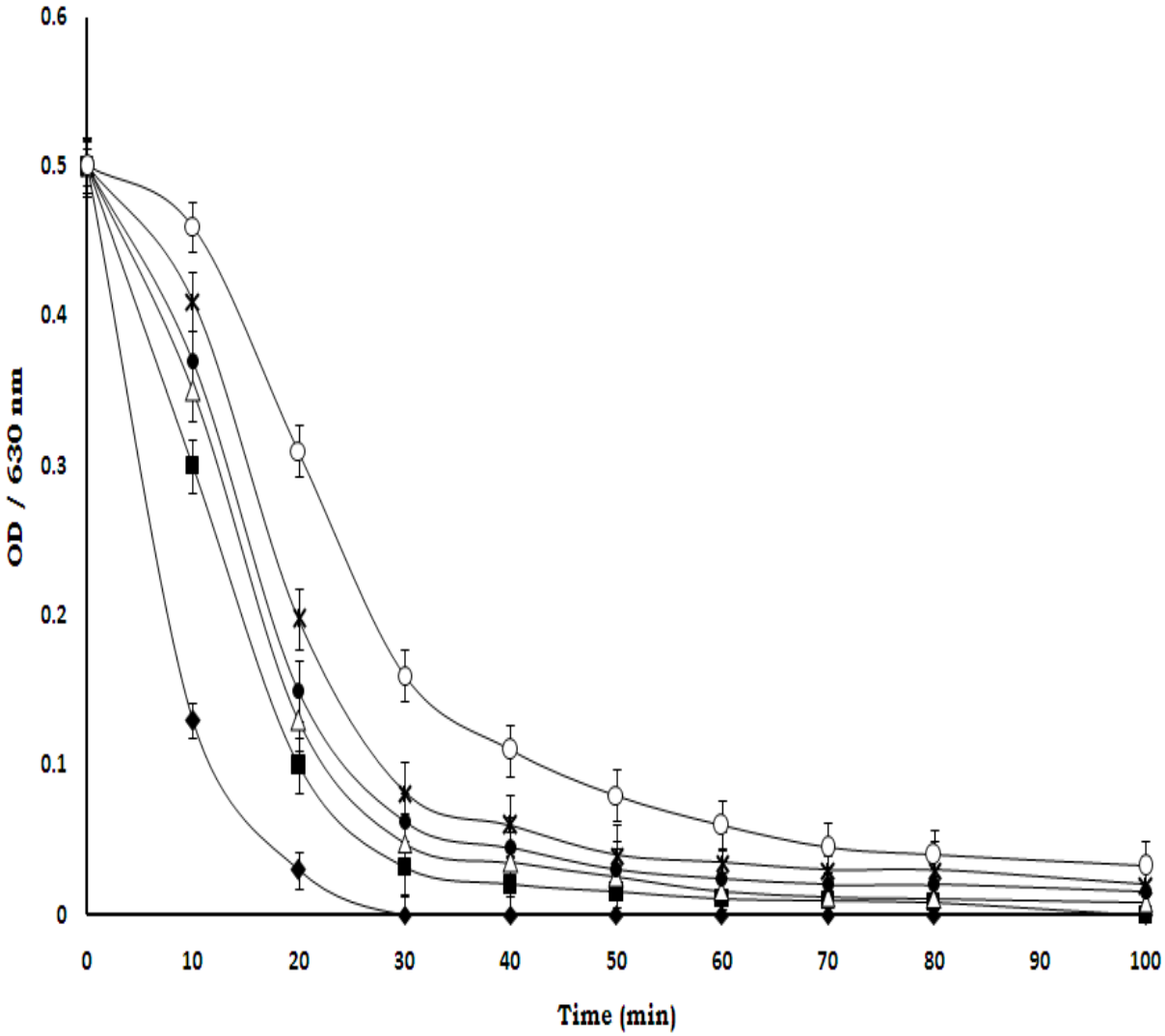
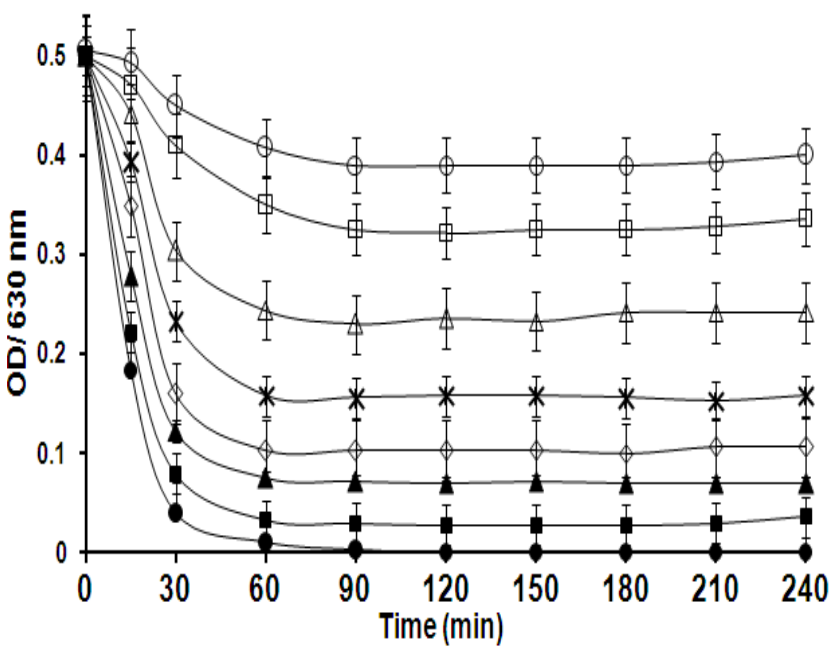
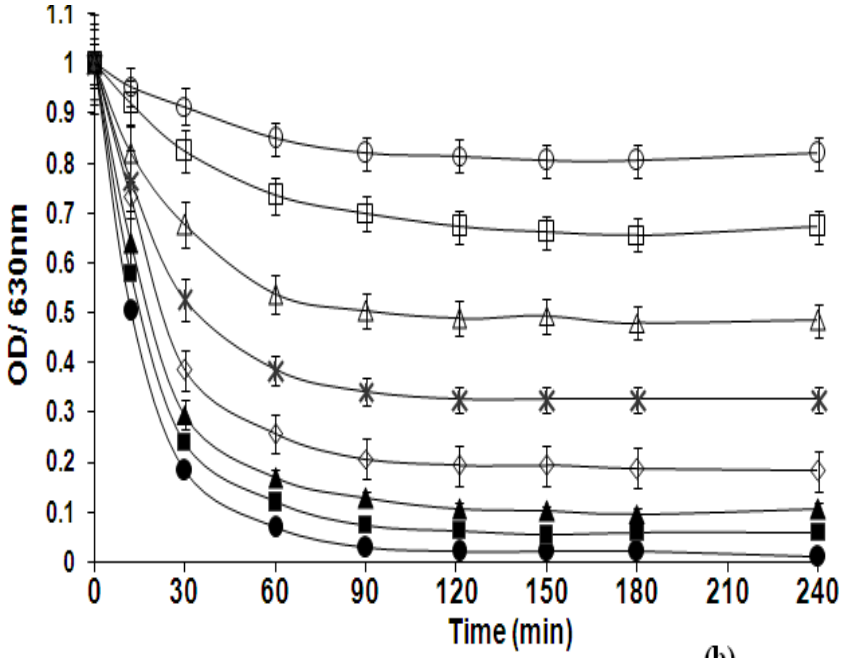


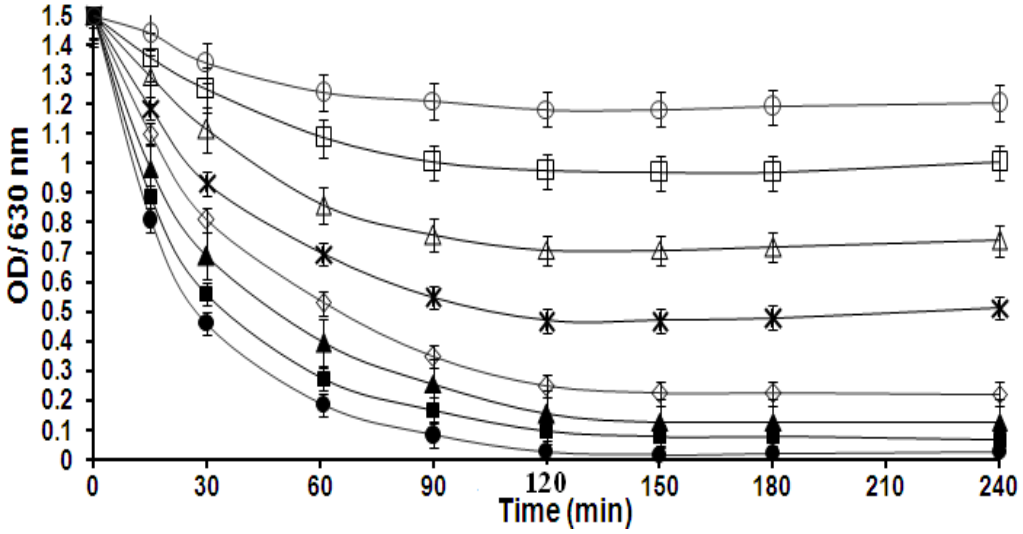
Figure 2


OD/ 630 nm
Time (min)
0
0.1
0.2
0.3
0.4
0.5
0
30
60
90
120
150
180
210
240

(a)


OD/ 630nm
Time (min)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
1.1
0
30
60
90
120
150
180
210
240

(b)


OD/ 630 nm
Time (min)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
1.1
1.2
1.3
1.4
1.5
0
30
60
90
120
150
180
210
240

(c)

Figure 3

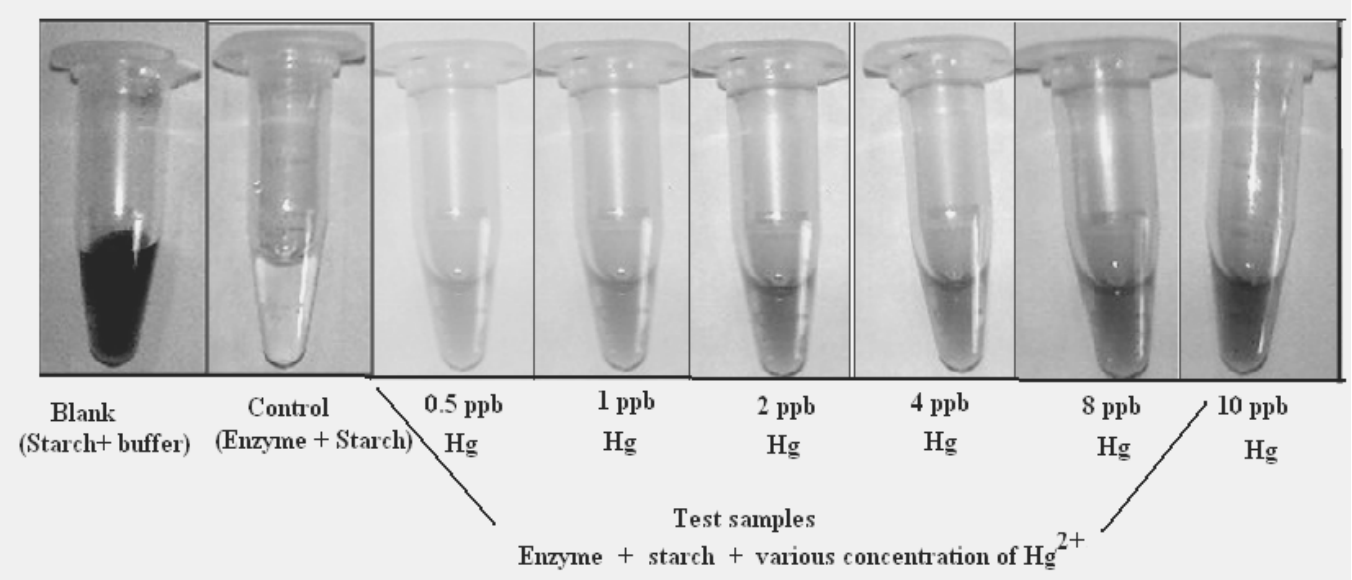
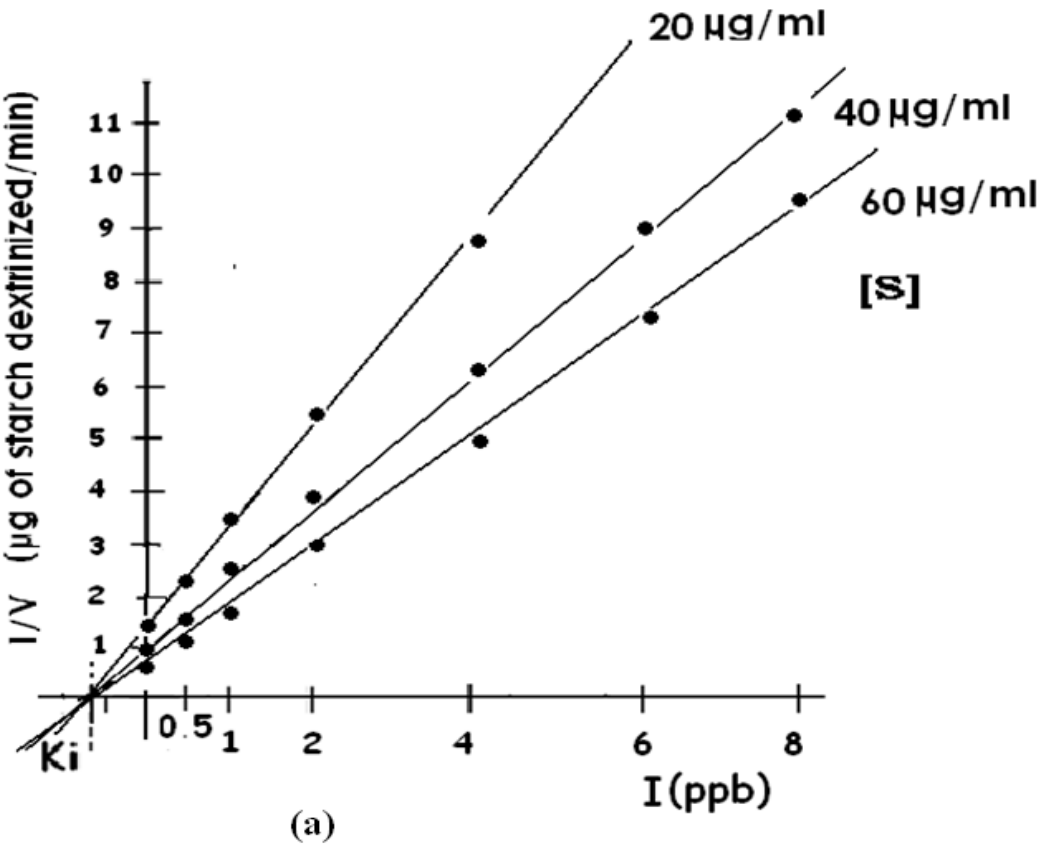

Blank
(Starch+ buffer)
Control
(Enzyme + Starch)
0.5 ppb
Hg
1 ppb
Hg
2 ppb
Hg
4 ppb
Hg
8 ppb
Hg
10 ppb
Hg
Test samples
Enzyme + starch + various concentration of Hg^{2+}

Figure 4


20 µg/ml
40 µg/ml
60 µg/ml
[S]
I/V (µg of starch dextrinized/min)
11
10
9
8
7
6
5
4
3
2
1
Ki
0.5
1
2
4
6
8
I(ppb)
(a)

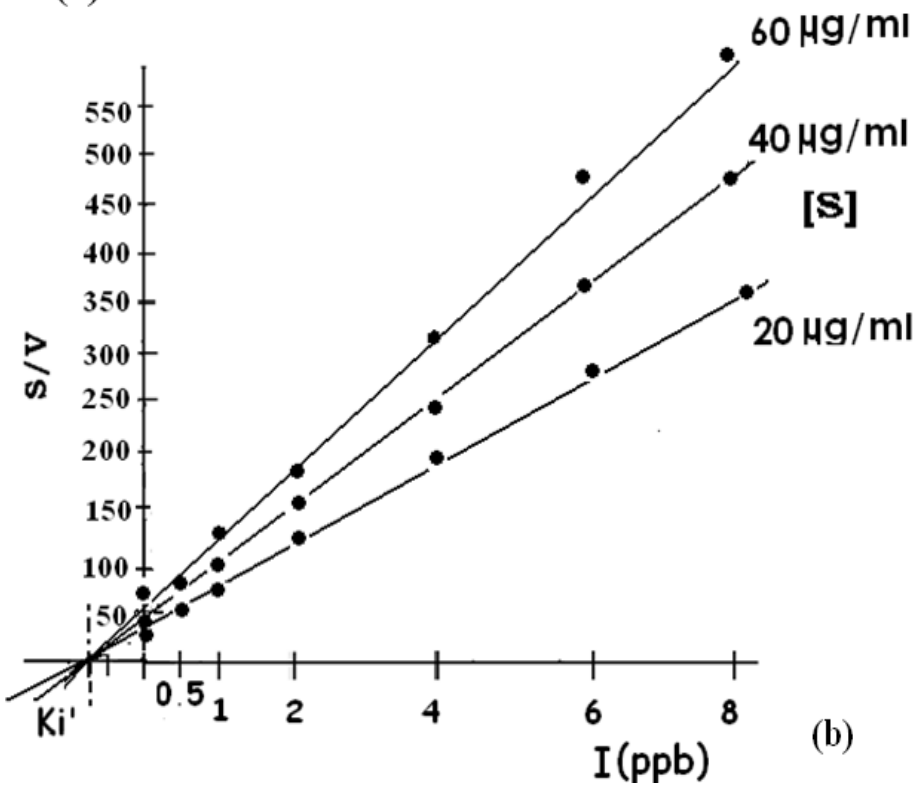

60 µg/ml
40 µg/ml
[S]
20 µg/ml
S/V
550
500
450
400
350
300
250
200
150
100
50
Ki'
0.5
1
2
4
6
8
I(ppb)
(b)

Figure 5

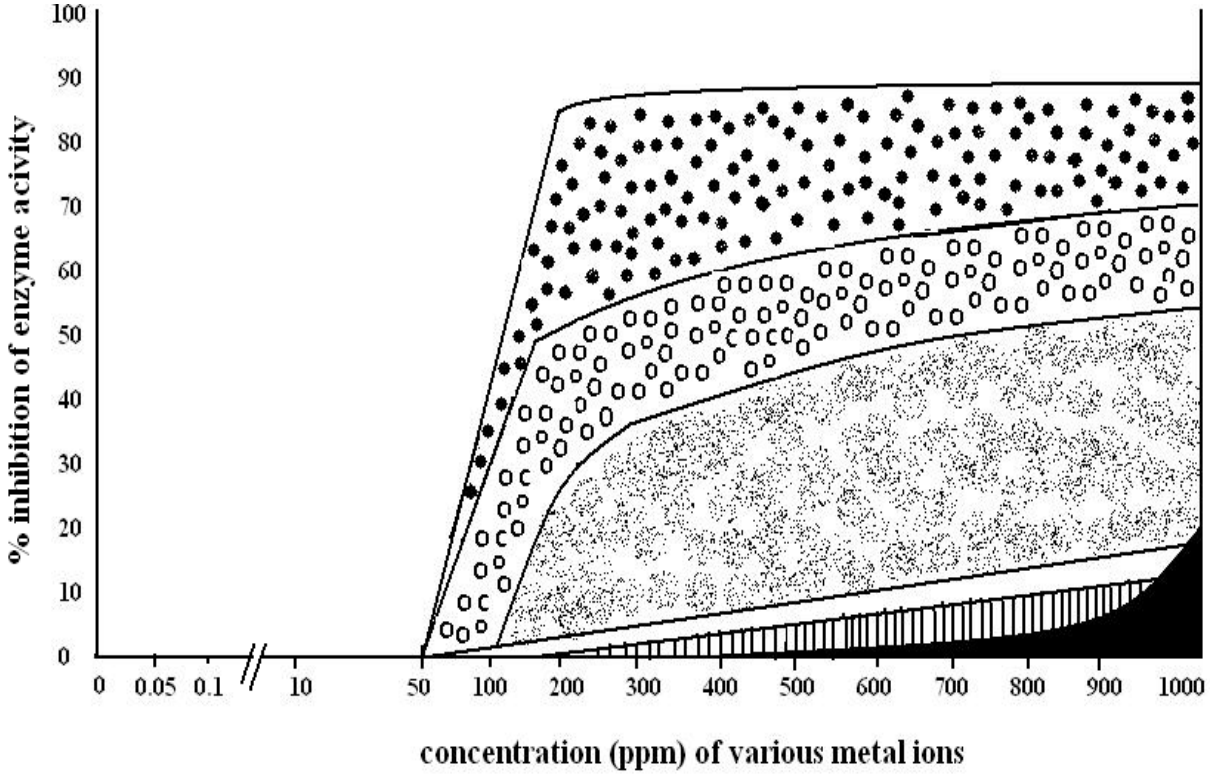

% inhibition of enzyme acivity
100
90
80
70
60
50
40
30
20
10
0
0
0.05
0.1
10
50
100
200
300
400
500
600
700
800
900
1000
concentration (ppm) of various metal ions

Figure 6