Effects of heavy metals ions for inhibition

The present research was carried out to performed analysis of jack bean urease by Hg+2 and Zn+2 ions after incubation of the enzyme with metal ions, as an inhibitor and without metal ion. Hg and Zn were studied as an inhibitor of jack bean urease in 20 mM phosphate buffer, pH 6. 5. The inhibition was carried out by the use of an incubation procedure in the presence of substrate urea. The influence of the inhibitor concentration and the incubation time on the enzyme activity was elucidated. It was found that increase in Hg and Zn concentration resulted in a linear decrease of urease activity. The inhibition became time independent in the studied time range. This observation is characteristic of a slow binding mechanism of inhibition. The protective experiment proved that the urease active site is involved in the binding of Hg and Zn. Urease may be utilized as a bioindicator of the trace levels of metal ions in environmental monitoring, bioprocess control or pharmaceutical analysis.

The enzyme urease is highly sensitive to trace metal ions. Different metal inhibit with quit different behavior in their ability to act as enzyme inhibitor. In the case of urease the Hg ion is an extremely efficient inhibitor, while Zn ion is relatively weak inhibitor (Wieslaawa et al., 2004). Urease inhibition was first studies with Hg+2 and Zn2+, since it has been reported in literature as being the most potent inhibitor for the urease enzyme, along with Cu+2 and Ag2+ (Krajewska, 1999; Andres and Narayanaswamy, 1995) . The non essential elements such as Pb and Cd are considered to be toxic and their presence in body can cause profound biochemical and neurological changes in the body even at ultra trace level. Primary sources of metal pollution are various metallurgical processes such as metal extraction, purification,

mining, smelting and refining. Among other sources, fossil fuel such as coal and oil release the metallic particles whereas, the most diverse source of pollution is the production of industrial products containing metals in various forms. These metallic pollutants follow many pathways to enter into the environment. In case of human body, the main routs of exposure to these metals are absorption through skin, direct inhalation and gastrointestinal absorption (Bermen and Talib, 1990)

The environmental toxicity of mercury is associated almost entirely with eating fish this source accounts for some 94 percent of human exposure. Surface reducing bacteria in sediments generate methyl mercury and release it into the water above, where it is absorbed by fish and passes across their gills or from their food supply (Thomas, 2004). Elemental mercury vapours can enter the body through inhalation and be carried by the blood stream to the brain, where it penetrates into the blood brain barrier. It disturbs psychopathological symptoms such as shyness, insomnia, depression and irritability. Divalent ionic mercury, Hg+2, damages the kidneys. Organometallic mercury compounds are also very toxic, dimethyl mercury, Hg (CH3)2, spectacularly so, killing most of the early investigators who fist synthesized it (Zelman et al., 1991). The maximum allowable level of any toxic vapor the level that is thought to be safe is called the threshold limit value (TLV) for mercury this concentration is set at 0. 05mg Hg per m3 air. All mercury spills are potentially very dangerous (Peter, 2004).

Zinc was recognized as an essential element in 1961. The human body contains 2. 3 gm of zinc which is more concentrated in the liver, prostate, voluntary muscles and bones (Arthur et al., 2003). The normal requirement

of zinc for an adult is 15 mg/day. Zinc deficiency leads to skin disease, impaired development of gonads, dwarfism, loss of appetite, anemia and loss of body hairs. Large excess zinc can cause convulsion, paralysis and even death (Bhattchaharya, 2005). Zinc deficiency leads to skin disease, impaired development of gonads, dwarfism loss of appetite, anemia and loss of hairs. Large excess of zinc can cause convolution, paralysis and even death. In some enzymes there may be two zinc centers present. One has the catalytic and the second may act as activator or inhibitor. It has been reported that the effect of metal ions on enzymes is usually due to their binding to thiol groups present in, or near, the active centre (Web, 1966) producing an irreversible inhibition (Zhylyak et al., 1995).

Enzymatic reactions have proved to be very promising tools to identify major pollutant such as heavy metals, enabling a very accurate toxicity identification evaluation (TIE) based on their inhibition. Assays based on the inhibition of urease show a high selectivity for the sensitive and effective based screening of heavy metals (Brack et al., 2000). Most urease inhibition assays are based on the measurement of either pH changes (Kormos and Lengauer, 2000; Krawczyk et al., 2000) or ammonia production (Soldatkin et al., 2000). A significant shortcoming of urease inhibition tests are based on measurement of ammonia evolution is the limitation to environmental samples with low content of ammonia (Jung et al., 1995). If the pH increase as an indicator of ammonia production is measured, a comparison of environmental samples is only possible if they have a uniform buffer capacity, which is mainly achieved by titration of the sample capacity, which

is mainly achieved by titration of the sample after incubation with urea and urease to the starting pH (Brack et al., 2000).

Owing to its pronounced sensitivity, urease has been considered as a primary enzyme for application as a probe for heavy metal ions. The sensitivity of urease to heavy metal ions is due to the presence of multiple cysteine residue, of which one, conserved principally to all known ureases, is located in the mobile flap of the active site of the enzyme (Todd and Hausinger, 1999). Therefore, an amperometric detection system for monitoring urease activity will significantly overcome the shortfalls listed above when using potentiomentry for urease activity measuring system. Also, an amperometric system based on screen-printed electrodes would allow the production of simple, inexpensive and portable devices for the rapid screening of heavy metals in the field. Such approach would allow the sensitive and accurate evaluations of heavy metals toxicity in environmental samples. Enzyme properties like its activity can be improved by some operations like site directed mutagenesis (Charles, E. O. 2003) [17]

In this work we investigated the inhibitory effect of Hg+2 and Zn2+ ions on jack bean urease. In order to define and characterize the mode of Hg+2 and Zn2+ enzyme interactions leading to the inhibition, we performed an analysis to measure the activity of enzyme after incubation of enzyme with metal ions and without metal ions, we investigated the inhibition of jack bean urease by Hg+2 and Zn2+. In contrast to most other studies where the observations were limited to the initial stage of reaction carried out in an enzyme-inhibitor pre-incubated system, we recorded the progress curves of the two reaction mixtures, with and without enzyme- inhibitor preincubation.

For both the ions we observed inhibition was time-dependent and could be best described by a slow binding mechanism (Wieslaawa et al., 2003). Both plants and microorganisms use the enzyme for decomposing a variety of environmental source of urea, urea fertilizers included, to provide ammonia as a nitrogen source. Eukaryotic ureases, among them plant urease from carnival ensiformis (jack bean), are homomeric with a typical composition, whereas prokaryotic ureases, such as those form Klebsiela aero genes and Bacillus pasteuni, are heteromeric with a typical timer of timers composition (Amin et al., 2006).

Materials and Methods:

Urease from jack bean type III, activity 45 units/ mg solid and urea for molecular biology, HgCl2 and ZnSO4 were purchased from Merck Germany. Two buffers (0. 01M) pH 5. 1 and 6. 8 were prepared by dilution methods. Ultrapure distil deionised water from Millipore water purification system was used throughout. The pH was adjusted by (0. 1M) NaOH and HCl solutions. The solutions of both the metal salts and urease were prepared in water, which was done to avoid the side reactions of metal ion with buffer that could interfere with their main reaction with urease. All the measurements were performed at room 250C ± 10C.

For inhibition were studies on jack bean urease and urea hydrolysis. Different concentrations of 1000, 700, 500, 300, 100 and 50 µl of Hg and Zn inhibitor were mix with urease enzyme and keep it for 10 mint for inhibitor completely react with urease at 37 0C. After added 20 mM solution of BCP and urea at pH 5. 1 and kept it again for 10 min. at same temperature. The remaining enzyme which is not inhibited by inhibitor reacts with urea. The color of the

mixture at this stage was purple which shows that enzyme is affected by inhibitor.

A simple bromocresol purple (BCP) method described by Yuh-Ling Lin, was adopted for the determination of Zn+2 and Hg+2 by the inhibition of urease. To the best of our knowledge this method has not been exploited before for the determination of Zn+2 and Hg+2. The method was first adopted for the optimization of different reaction conditions, and extended for urease inhibition study.

Principle:

In this assay pH change plays an important role. In the urease-catalyzed reaction, urea is converted to NH3 and CO2. The NH3 released during the reaction increases the pH of reaction mixture, which is immediately detected by the pH indicator, bromocresol purple, already present in the reaction mixture. It changes colour with the change of pH (transition stage of BCP is 5. 2 to 6. 8); it shows reddish yellow colour at pH 5. 2, which changes to purple at pH 6. 8 which absorbs at 588nm.

Inhibition studies of urease Enzyme:

The enzyme activity was measured through its catalytic effect on the hydrolysis of urea. The enzymatic activity was related with the optical density (OD) or absorbance (As) recorded at 588 nm. In order to determine the optimum conditions for the assay according to—– and parameters were analyzed and studied for inhibition, incubation time for absorbance, temperature, enzymatic activity ( ) and substrate condition at optimum conditions. The time for the hydrolysis of urea into NH3 and CO2 was found

20 mints. Most of the enzymes denature above 40 0C, we found that the optimum temperature for urease is 370C. By increasing the enzyme volume the reaction rate also affected because substrate amount remains constant and urease amount increases as s result reaction rate boost up to a certain limit. Similarly when we kept the urease volume constant and increase the urea concentration, as a result of this more urea is converted to NH3 and CO2. But if we increase the urea concentration the reaction rate became slow.

Results and Discussion

Metals Effects on Urease activity:

During standardization, to investigate the time period for a sufficient reaction to take place the enzyme was incubated with substrate (0. 1M) for 1 – 22 minutes. A time period of 20 minute was found to be sufficient for maximum hydrolysis of urease. During this study, it was also found that the optical density of the reaction mixture shows no increase as the reaction goes towards equilibrium with the passage of time. Like most chemical reactions, the rate of an enzyme-catalyzed reaction increases as the temperature is increased. In the case of enzymatic reactions, this is complicated by the fact that enzymes are adversely affected by high temperatures, the reaction rate increases with temperature to a maximum level, than abruptly declines with further increase of temperature. Because most enzymes are rapidly denatured at temperatures above 40oC, hence enzymatic determinations are usually carried out somewhat below this temperature. In order to find out optimum temperature, for the activity of urease, a temperature range of 25 – 45oC was studied and 37 oC was found

most suitable for enzyme reaction. There is an increase in enzyme activity up to 37oC and then a decline at and above 40oC.

Effect of urease concentration on its activity

In order to study the effect of enzyme concentration (by increasing volume of enzyme solution) on the reaction rate, keeping the substrate concentration constant, so as the reaction should be independent of the substrate concentration. Any change in the absorbance is related with the amount of product formed in a specific time period, and was dependent on the volume of enzyme solution added to the reaction mixture. Effect of increasing urease concentration on reaction rate was studied. An increase in absorbance with increasing volume of enzyme solution was observed, as shown in Fig. 3. Volume of enzyme for further studies was selected 20 µl because at this volume the absorbance was sufficient for study and the effect of buffered enzyme solution was very small on the pH of the reaction mixture.

Effect of urea on urease activity

During the optimization of assay, effect of substrate volume (i. e. urea) on the enzyme activity was studied for the given volume of enzyme (20 µL). A series of reaction mixtures containing from 0. 1 – 0. 50ml of 0. 1M urea solution were studied. An increase in reaction rate was observed as the volume of urea solution was increased up to a certain level after that there was no significant change. During the optimization of assay conditions, effect of substrate concentration (i. e. urea) on the reaction rate with the passage of time was studied. A series of reaction mixtures containing 1 ml of urea from urea solutions ranging 0. 83 – 16 mM were studied. An increase in

reaction rate was observed with increasing urea concentration and incubation time. The concentration of urea 20 mM was found best for inhibition study.

Effect of Hg2+ and Zn2+ on urease inhibition

Various concentrations of Hg+2 and Zn+2 were prepared in distilled deionized water (pH 5. 1). In the first set of experiment absorbance for standard solution (As) of urea was recorded at 588nm under optimized assay conditions. In the second set of experiment various volumes of (50-1000 µl) standred Hg+2 of the concentration 1×10-4M were with urease for ten minutes prior to the addition of urea and BCP in five different test tubes. The absorbance in the presence of inhibitor (Ai) was recorded in the similar fashion. The absorbance for different concentrations of Hg+2 studied by increasing the inhibitor volume so absorbance decreased as Hg2+ inhibit the urease. From the above data the %age inhibition was calculated by the formula. The standard graph between %age inhibition and various volumes (µl) of Hg2+is shown in Figure 8. The results indicate that Hg+2 is the potential inhibitor of urease and 50µl of solution of Hg+2 gave 54% inhibition. With limited optimization of reaction conditions, only limited range of Hg+2 concentrations were studied and graph was linear in this range. However sensitivity of method could be improved by further optimizations.

Same sets of reaction conditions were exploited for the determination of Zn+2. The volumes of Zn+2 solutions used are 50-1000µl. The absorbance (Ai) against each volumes of Zn+2 with 1×10-5 M conc. And graphical representation of absorbance under different volumes of Zn+2 is in Figur8. The %age inhibition of urease is also calculated for the different

concentrations of Zn+2 by using the same formula. The graph is linear in the range of inhibitor concentration studied. The results indicate that Zn+2 is also a potential inhibitor of urease like Hg+2. The results clearly demonstrate that the method can be successfully exploited and extended for the determination of Hg+2 and Zn+2 in the real samples. After further optimization of reaction conditions, limit of detection could be improved.

Conclusion

The method proves to be economical, easy and simple. The sensitivity of the method would encourage future researchers to exploit in for the demonstration of heavy metals in real samples. The method can be used as a tool to detect trace metal pollutants in the environment.

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