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Development of Fluorescence based Biosensor for Estimation of Heavy Metal Ions

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***Abstract*—For the first time, acid phosphatase based fluorescence biosensor has been developed for estimation of heavy metal ions. It is based on inhibition of acid phosphatase enzyme activity exerted by metal ions. Acid phosphatase entrapped A-J biocomposite membranes have been employed for the development of fluorescence biosensor. The extent of inhibition for different toxic metal ions was studied by measuring the decrease in fluorescence intensity. The results indicate that the toxicity of the various metals tested toward immobilized phosphatase is ranged as follows: Hg^{2+} >Cu^{2+} > Cr^{2+}. The storage stability of the enzyme at 40C was found to be more than two months. Generally, in inhibition based biosensor reuse of bioelement is quite difficult. In present studies regeneration of ACP membrane has been achieved successfully.**

Keywords-biosensor; fluorescence; heavy metal ions; agarose; guar gum

I. INTRODUCTION

Heavy metals like Cu^{2+}, Cd^{2+}, Cr^{2+}-, Hg^{2+}-, Pb^{2+} and Zn^{2+} are very toxic and tend to bioaccumulate in living organisms, especially in marine organisms[1].Because they are non-biodegradable, can be a serious threat to the environment and human health. Metallic constituents of pesticides and therapeutic agents, burning of fossil fuel containing heavy metals, mining, tanning and chemical manufacturing industries are the major sources of heavy metal poisoning [2]. Considering their adverse effects on human health and overall environment, there is urgent need to monitor heavy metal ions. Conventional techniques for heavy metal analysis include inductively coupled plasma mass spectroscopy, X-ray absorption spectroscopy, cold vapour atomic absorption spectroscopy and UV visible spectrophotometry [3]. Though precise, these methods are costly and require trained personnel. Also they are laboratory bound. Therefore biosensors are becoming popular in this regard. Various enzymes like urease [4], glucose oxidase [5], acetyl cholinesterase [6], invertase [7] with different transducers have been employed for the estimation of heavy metal ions.

Enzymatic methods are commonly used for metal ion determination, as these can be based on the use of a wide range of enzymes that are inhibited by low concentrations of certain metal ions. Apart from enzymes different bioreceptors like antibody [8], whole cell [9] and genetically engineered microorganisms [10] have been employed for the detection and estimation of heavy metal ions. There are very few reports where phosphatase enzyme has been employed for the construction of fluorescence based biosensor.

Durrieu and Tran-Minh [11] developed an enzyme optical fibre based biosensor for the detection of heavy metals that employed alkaline phosphatase present on the outside membrane of live Chlorella vulgaris microalgae. F. García Sánchez *et al* reported the fluorescence biosensor for Ag^{+} and CN^{-} based on alkaline phosphatase [12].

In this study, we have attempted to fabricate fluroscence biosensor for the detection and estimation of heavy metal ions. Biosensor is based on the inhibition of acid phosphatase. There are very few reports where phosphatase enzyme has been employed for the construction of fluorescence based biosensor. It is the first report on acid phosphatase based fluorescence biosensor for the determination of heavy metal ions.

II. EXPERIMENTAL

A. Materials and reagents

1-naphthyl phosphate, mercuric chloride, cupric acetate , chromium chloride and agarose were procured from Sisco Research Laboratory (SRL) India. All other chemicals were of analytical grade, and all solutions were prepared with water from the Millipore Milli-Q system. Jellose was isolated in our laboratory.

B. Extraction and partial purification of acid phosphatase enzyme

The enzyme acid phosphatase was extracted from the seeds of *Phaseolus vulgaris* by using acetate buffer saline (pH 5.5, 0.1 M). It was further partially purified by 70%

ammonium sulphate salt saturation concentration and DEAE-ion exchange chromatography.

C. Entrapment of acid phosphatase in agarose - jellose membrane

1 ml of 3% agarose was mixed with equal volume of 1% jellose and the solution was allowed to cool to ~ 40°C. Then partially purified ACP (25 µg) added to this mixture and thoroughly mixed and cast on a plastic sheet (2.4x3.7cm^2) supported by glass slide. It was allowed to dry at 37°C. The membrane was slowly and carefully detached from the plastic support and stored under refrigeration in order to prevent any bacterial growth on the membrane. The membrane containing ACP, thus prepared, was cut into pieces of dimensions 0.8cm X 0.8cm and these pieces were used for biosensor fabrication.

D. Instrumentation

The fluorescence studies were carried out on Shimadzu RF-5301 PC spectroflurometer.

E. Construction of fluorescence biosensor

Acid phosphatase in acidic medium catalyzes the conversion of 1-naphthyl phosphate to 1-naphthol a highly fluorescent product having λex 346 nm and λem 463 nm.

The construction of biosensor was carried out by placing thin, transparent and flexible membrane of entrapped ACP at the bottom of the fluorescent cuvette containing acetate buffer (pH 5.5, 0.1 M). Subsequent addition of substrate 1-naphthyl phosphate was carried out. Afterwards, reaction was arrested by adding 1 ml Tris-HCl (pH 9, 1M). Change in the fluorescence intensity due to the formation of 1-naphthol was recorded at 463 nm. A blank spectrum was also recorded for the solution containing Tris-HCl (pH 9, 1M), substrate 1-naphthyl phosphate and ACP entrapped membrane, added in the same sequence.

F. Determination of kinetic parameters for free and immobilized ACP

A piece of acid phosphatase immobilized membrane (0.8 x 0.8 cm^2) consisting of 1.8 µg was taken in cuvette containing acetate buffer (pH 5.5, 0.1 M) and incubated for 5 minutes. Thereafter, substrate 1- naphthyl phosphate (3.3 x 10^{-6} M to 2.3 x 10^{-5} M) was added and incubated for 10 minutes. The reaction was arrested by the addition of 1 ml of Tris-HCl buffer (1 M, pH 9.0). The product thus formed was measured at λem 463 nm. Amount of product formed was determined by extrapolating intensity on standard 1-naphthol curve.

Same assay as mentioned above with 2 µg (19 U) of enzyme was used to determine Km and Vmax for free enzyme. A graph of velocity (V) Vs substrate concentration (S) and a double reciprocal plot (Lineweaver-Burk plot) i.e., 1/V Vs 1/(S) were plotted. From this graph Km and Vmax for free and immobilized ACP were determined.

G. Estimation of heavy metal ions by inhibition based fluorescence biosensor

The estimation of heavy me was achieved by performing the assay as mentioned above except initially incubation of enzyme membrane was carried out with varying concentration of Hg^{2+} ranging from (16.6 x 10^{-6} M to 83x10^{-6} M) for 5 minutes. Subsequent addition of 1-naphthyl phosphate (1.66 x 10^{-5} M) was carried out followed by incubation at room temperature for 10 minutes. Inhibitor blank was prepared in the same way except addition of Hg^{+2} and fluorescence intensity was measured at 463 nm.

A graph of % inhibition Vs concentration of inhibitor was plotted to find the linear range. In the similar way calibration curve for Cu^{2+} (3.33 x 10^{-5}M – 1.66 x10^{-4} M) and Cr^{+2} (8.3 x 10^{-5} M - 5.05 x 10^{-4} M) was obtained.

III. Results And Discussion

The acid phosphatase enzyme purified fraction showed 38 fold purification with 5% yield. Immobilization is an important step in the fabrication of biosensor; therefore acid phosphatase has been immobilized in the blend of biopolymers. For the first time acid phosphatase has been successfully immobilized in a composite of agarose-jellose with 66% retention of enzyme activity. The maximum retention of enzyme activity with negligible leaching was observed due to the fine tuning of porosity achieved by mixing two polysaccharides in desired proportion. Comparable Km and Vmax values (Table I) of free and immobilized enzyme shows that there is no change in the conformation of enzyme as well as no constraint on the diffusion of substrate and subsequent release of product.

TABLE I. Determination Of Kinetic Constants Of Acp Enzyme

Enzyme	Km(µM)	Vmax (µmole/min)
Free ACP	1.47	0.35
Immobilized ACP	1.66	0.28

A. Optimization of substrate concentration for fluorometric assay

A membrane of immobilized enzyme of dimension 0.8 x 0.8 cm^2 was chosen to optimize the substrate concentration for inhibition studies since it contains approximately 2 µg of enzyme, which is approximately equal to that of free enzyme.

In the procedure adopted in this work the enzyme substrate reaction is initiated after a preincubation of the sensor with heavy metal ions. Therefore, high substrate concentrations can then be utilized for enzyme inhibition assay as it avoids the problem of having little fluroscence intensity at low substrate concentrations. In view of this, 1.66 x 10^{-5} M concentration of 1-naphthyl phosphate is used

for the further measurements of inhibition of acid phosphatase.

B. Estimation of heavy metal ions by fluorescence sensor

The effect of Hg^{2+}, Cu^{2+} and Cr^{2+} on acid phosphatase activity was studied by constructed ACP based biosensor. A Fluorescence spectrum for inhibitory effect of Hg^{2+} on acid phosphatase activity is depicted in Fig. I. The spectra revealed that florescence intensity is inversely proportional to inhibitor concentration, since increase in heavy metal concentration leads to increase in inhibition of enzyme and consecutive release of a reduced amount of product. A linear range for fluorescence biosensor for Hg^{2+} was obtained in the range 16.6 µM -83.33 µM with lower detection limit 8.33 µM.

The effect of Cu^{2+} and Cr^{2+} on acid phosphatase activity was studied by incubating the immobilized acid phosphatase membrane with increasing concentration of heavy metal ions. Linear range, lower detection limit and I_{50} value is given in Table II.

TABLE II. ESTIMATION OF HEAVY METAL IONS BY ACP BASED BIOSENSOR

Metals	Linear range µM	Lower detection limit µM	I_{50} Value µM
Hg^{+2}	16.6-83.33	8.33	103.55
Cu^{+2}	33.3 – 166 .66	33.3	208
Cr^{+2}	83.3 - 505	50	416

Lower detection limit of the present fluorescence biosensor is 33.3 µM for Cu^{2+} which is slightly lower than the array based optical biosensor based on inhibition of urease and acetylcholinesterase. Lower detection limit of this biosensor is 50 µM for Cu^{2+} [15].

There are no reports where acid phosphatase has been employed with fluorescence transducer for estimation of heavy metal ions. García [42] reported the alkaline phosphatase based fluorescence biosensor for Ag^{+} with linear range 15-152 µM and detection limits of 10.1 µM.

C. Reusability of fluorescence based ACP biosensor

Heavy metals generally inhibit the enzyme activity by binding the metal salts to thiol or methyl thiol groups of enzymes.

Reactivation of the heavy metal ions inhibited enzyme in the biosensor has been achieved by using metal chelating agents, such as EDTA or thiols [13]. Mohammadi *et al.* [14] tried to regenerate a 50% mercury-inhibited invertase biosensor by soaking in a cysteine solution; the recovery was 30% of the initial biosensor signal. In the current reactivation of the bioelement was gained by immersing it into 6 mM L-Cysteine solution. A full and rapid restoration of response has been achieved for 36% inhibited acid phosphatase biosensor for 13 consecutive cycles.

D. Inhibition by mixed heavy metal ions

The effect of mixture of heavy (33.2µMHg^{2+}, 33.2µM Cu^{2+} and 83.3µM Cr^{2+}) metal ions on immobilized ACP was studied as preliminary investigation towards its application for sensing of total heavy metal ions.

It is observed that mixture of heavy metal ion exhibit additive effect on the performance of biosensor which demonstrates the suitability of the biosensor for determination of total heavy metal ions.

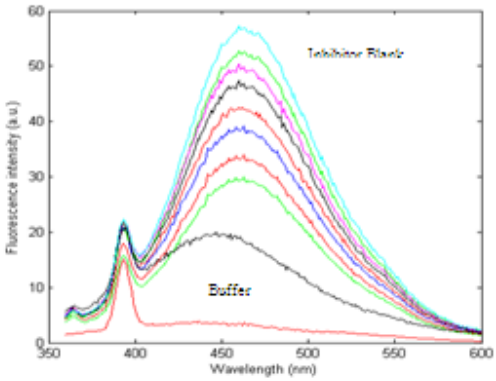

Figure 1. Fluroscence spectra for inhibition of acid phosphatase by Hg^{2+} (16.6 µM - 83.33 µM))

IV. CONCLUSIONS

The work has demonstrated that a simple and easy to construct biosensor can be developed for monitoring trace heavy metal ions. For the first time, acid phosphatase inhibition based fluorescence biosensor has been developed for estimation of heavy metal ions. Biosensor is based on inhibition of ACP activity exerted by metal ions. The extent of inhibition for various toxic metal ions was different.

However, since the proposed biosensor is inhibition based sensor, it cannot discriminate among different inhibitors of acid phosphatase. Apart from the analysis of heavy metal ions, the inhibition based biosensor presented in this work might also be useful for the determination of other biologically active compounds, such as carbamate pesticides, organophosphorous pesticides etc.

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