A chemiluminescence-based biosensor for metal ion detection

Sanjay D. Kamtekar a, Rajiv Pande a, Madhu S. Ayyagari a,b, Kenneth A. Marx c, David L. Kaplan b, Jayant Kumar d, Sukant K. Tripathy a,*,

a Center for Advanced Materials, Department of Chemistry, University of Massachusetts, Lowell, MA 01854, USA
b Biotechnology Division, US Army Natick RD&E Center, Natick, MA 01760, USA
c Center for Intelligent Biomaterials, Department of Chemistry, University of Massachusetts, Lowell, MA 01854, USA
d Center for Advanced Materials, Department of Physics, University of Massachusetts, Lowell, MA 01854, USA

Abstract

Inhibition of the native metalloenzyme, alkaline phosphatase, in the presence of some metal ions, and the reactivation of its apoenzyme by Zn(II) ions is used to determine metal ion concentrations. Alkaline phosphatase-catalysed hydrolysis of a chemiluminescent substrate, chloro 3-(4-methoxy spiro[1,2-dioxetane-3-2'-tricyclo[3.3.1.1]-decan]-4-yl) phenyl phosphate, generates light. By measuring the chemiluminescence signal strength in the presence or absence of metal ions, this reaction can be used to detect and determine metal ion concentrations. The immobilization of alkaline phosphatase on different glass surfaces by covalent coupling using a bifunctional reagent, glutaraldehyde, was demonstrated. Using chemiluminescence measurements, Zn(II), Be(II) and Bi(III) were detected in trace levels. This technique forms the basis in the development of a metal ion-based fibre optic sensor.

Keywords: Alkaline phosphatase; Metal ion detection; Chemiluminescence; Fibre optics

1. Introduction

Trace analysis of heavy metals is important in the chemical, environmental and biomedical fields. Chemical and biochemical methods using optical or electrochemical techniques of signal transduction to detect metals have been studied [1-5]. Biochemical means of detection of metal ions often involve metalloenzymes, which require metals as cofactors for their enzyme activity [6].

Alkaline phosphatase (EC 3.1.3.1), a non-specific phospho monoesterase, is a dimeric metalloenzyme containing four zinc ions and two magnesium ions coordinated to the active site [6]. Although the Zn(II) ion is necessary for enzyme activity, excessive amounts of the ion result in the inhibition of enzyme catalytic activity [7-9]. Moreover, some other metal ions, such as Be(II) and Bi(III), are also known to be potent inhibitors of alkaline phosphatase [7-9]. Removal of metal ions from the native enzyme with strong chelating agents or by partial denaturation of the enzyme results in the formation of the corresponding apoenzyme that lacks catalytic activity. By exposure to a Zn(II) ion-containing solution, the apoenzyme can be reversibly activated [8-10]. The restored enzyme activity is proportional to the stoichiometric amount of Zn(II) ion present in the solution, and this measure of activity allows the evaluation of the metal ion content [8-10]. It is thus possible to develop sensitive methods for the determination of Zn(II) (in stoichiometric excess), Be and Bi based on enzyme inhibition. The presence of Zn(II) can also be determined by the apoenzyme reactivation.

Zn(II) ions in trace levels (p.p.b. range by enzyme reactivation and p.p.b. to p.p.m. range by enzyme inhibition) in bulk solutions have been determined by the present authors [9]. In addition to Zn(II), Be(II) and Bi(III) were also determined quantitatively. The technique involved the measurement of the chemiluminescence signal generated by the alkaline phosphatase-catalysed dephosphorylation of chloro 3-(4-methoxy spiro [1,2-dioxetane-3-2'-tricyclo[3.3.1.1]-decan]-4-yl) phenyl phosphate (CSPD), in the presence and absence of metal ions. As described earlier, the assembly and alignment of optical components for the detection system were simple because of in situ light generation in the reaction mixture [11]. In this paper, the same approach has been extended by immobilizing alkaline phosphatase on a glass surface via covalent bonding using a bifunctional reagent, glutaraldehyde. Upon introduction of the substrate solution, light generation was observed on the glass surface (for example, on the inside walls of a glass capillary) due to enzymatic
dephosphorylation of CSPD. The strength of the chemiluminescence signal is directly proportional to the enzyme activity at a given chemiluminescence substrate concentration.

2. Experimental details

2.1. Materials

Alkaline phosphatase (bovine calf intestine), Tris-HCl, ammonium sulphate (enzyme grade), zinc sulphate, p-nitrophenyl phosphate, 3-aminopropyltriethoxysilane and glass beads (200 μm) were supplied by Sigma Chemical Company (St Louis MO), while sodium acetate, sodium chloride and magnesium chloride were purchased from Fisher Scientific (Fair Lawn NJ). Beryllium sulphate, glutaraldehyde and bismuth nitrate were purchased from Aldrich Chemical Company (Milwaukee WI). Diethylamine (DEA) and CSPD were supplied as a part of Southern-Light Chemiluminescent Detection System by Tropix, Inc. (Bedford MA). CSPD was supplied as a 25 mM aqueous solution. Sapphire IITM, a luminescence amplifying material (referred to as enhancer in the text), was also supplied by Tropix, Inc. All chemicals were of analytical grade and were used as received.

2.2. Methods

Preparation of apoenzyme: apo-alkaline phosphatase was prepared by dialysing the native enzyme (10 mg ml⁻¹, in phosphate buffered saline, pH 7.2) against 2 l of 2 M ammonium sulphate, pH 9, at 4 °C for 24 h with two changes of dialysate. Ammonium sulphate was removed subsequently by dialysing twice against 2 l of 0.01 M Tris-HCl, 0.01 M sodium acetate, 0.1 M sodium chloride, pH 9 [12]. Prior to CSPD hydrolysis reactions, the native and apoenzyme activities were checked by following the hydrolysis of p-nitrophenyl phosphate (in 0.2 M Tris-HCl buffer, pH 8). The initial rate of formation of p-nitrophenol was recorded at 405 nm using a Perkin-Elmer Lambda 9 spectrophotometer. Zn(II) ion concentrations in the native and apoenzyme were checked by direct current plasma (DCP) spectrometry (model SMI III Spectrametrics Inc.) [13]. Stock solutions of Zn(II) and other metals were prepared by dissolving appropriate amounts of spectral grade salts in distilled deionized water. Further dilutions were made in DEA.

Immobilization on glass surface: glass beads and the inner surfaces of test tubes and 100 μl glass capillaries were silanized by treatment with 10% aqueous 3-aminopropyltriethoxysilane, pH 3.5, at 75 °C for 3 h. After silanization, the alkaline phosphatase-catalysed hydrolysis of CSPD under appropriate conditions generates light. A typical chemiluminescence signal profile from the CSPD hydrolysis is shown in Fig. 2A for two cases: (a) in the absence and (b) in the presence of a metal ion in solution. Fig. 2B gives the same

washed with distilled water and phosphate buffer after reducing the methine groups (formed during the coupling of aldehyde group with enzyme amino group) by incubating the enzyme–glass conjugate for 2 min in the immobilization supernatant containing 200 mg NaBH₄ [14]. Repeated alternate treatment of the silanized glass with glutaraldehyde and alkaline phosphatase can help build up an increasing mass of enzyme on the glass surfaces [15]. In the present study, alkaline phosphatase was used, as illustrated in Fig. 1. The immobilized alkaline phosphatase was stored at 4 °C in assay buffer. A packed bed of glass beads with covalently immobilized enzyme was also prepared in a simple Pasteur pipette in preliminary attempts to develop a continuous flow cell. The immobilized enzyme on beads retained its activity for well over six months when stored in assay buffer at 4 °C.

Substrate solution was prepared by adding the supplied 25 mM CSPD solution to a 10% solution of enhancer in assay buffer to give a final CSPD concentration of 0.4 mM. The stock solutions, stored at 4 °C, were brought to room temperature prior to the reaction. The reaction mixture was prepared separately by adding the assay buffer to predetermined volumes of CSPD and metal ion solution to make up the final volume. The reaction was initiated by adding 0.5 ml of this solution to a glass test tube or a 100 ml capillary containing the immobilized enzyme.

For the detection of Be(II), Zn(II) and Bi(III) by enzyme inhibition, the reaction mixture was prepared by mixing 0.25 ml of 0.4 mM CSPD solution (10% enhancer) and 0.125 ml of metal ion solutions of different concentrations, and then adjusting the volume to 0.5 ml with buffer. The reaction was initiated by placing this mixture in a test tube in which alkaline phosphatase had been immobilized. Zn(II) detection by reactivation of the apo-alkaline phosphatase was carried out by first incubating 0.5 ml of the metal ion solution in a test tube with immobilized apoenzyme for 2 min. To this, 1 ml of buffer and 0.5 ml of 0.4 mM CSPD solution were added to initiate the reaction.

A photomultiplier tube, an amplifier, a photon counter and a personal computer were used to collect and process the data. The experimental setup has been described in greater detail earlier [11]. Less than five seconds elapsed between the initiation of the reaction and the start of data acquisition. The signal reaches its peak value in about 3 to 5 min. The enzyme activity, proportional to the initial slope (counts s⁻¹), was calculated from the data points collected in 20 s. The data was normalized to the control (i.e. no inhibition) results for inhibition studies.

3. Results and discussion

Alkaline phosphatase-catalysed hydrolysis of CSPD under appropriate conditions generates light. A typical chemiluminescence signal profile from the CSPD hydrolysis is shown in Fig. 2A for two cases: (a) in the absence and (b) in the presence of a metal ion in solution. Fig. 2B gives the same
profile for the enzyme in the immobilized state. As explained in the methods section, the initial slope of these profiles was used as a measure of the reaction rate.

Immobilized alkaline phosphatase shows a similar chemiluminescence signal profile to that in bulk solution under similar experimental conditions. However, the signal intensity with immobilized enzyme is weaker, perhaps due to relatively fewer numbers of enzyme molecules (calculated on the basis of complete surface coverage by the immobilized enzyme) catalysing the reaction. It is also possible that the enzyme may have partially lost its activity during the process of immobilization. In order to enhance the initial burst of photon release, the number density of enzyme on the glass surface could be increased by repeated alternate treatments of the surface with glutaraldehyde and the enzyme.

3.1. Detection of Be(II), Zn(II) and Bi(III) by the inhibition of immobilized alkaline phosphatase

The inhibition of alkaline phosphatase in the presence of Be(II), Zn(II) and Bi(III) was studied to quantify the metal ion concentrations. As described in Section 2, the enzyme was immobilized on the inside walls of a test tube. Data collection for successive lower metal ion concentrations was continued until the detection limit was reached, i.e. the point where the difference in the initial slopes for the lowest metal ion concentration and the corresponding control (absence of any metal ion inhibitor) is indistinguishable. The error bars in the figures signify the standard deviation of three different samples taken at the same metal ion concentration. Figs. 3(a), (b) and (c) show the calibration curves obtained for Be(II),
Zn(II) and Bi(III) respectively. The detection of 5 p.p.b. Be, 120 p.p.b. Zn and 2.4 p.p.m. Bi was achieved with immobilized alkaline phosphatase. These detection limits are comparable to our solution data and to the values reported in the literature [1,3-10]. The detection of these three metal ions can be made more specific by using different masking agents and by sample pre-treatment [7-9]. Determination of Zn(II) in the presence of Be(II) by selective masking of Be(II) using acetylacetone and sodium fluoride has been demonstrated in earlier studies [9]. Acetylacetone effectively masks silver, nickel, cobalt and copper ions along with beryllium, and fluoride masks the effect of aluminium, strontium, calcium and manganese if these interfering metal ions are present in the sample [7,16]. Investigations are currently under way to develop sample pre-treatment protocols for selective metal ion detection.

Fig. 2. A: Chemiluminescence signal profile of alkaline phosphatase-catalysed dephosphorylation of CSPD (a) in the absence and (b) in the presence of Zn(II) 100 p.p.b. in solution. B: Chemiluminescence signal profile of alkaline phosphatase-catalysed dephosphorylation of CSPD (a) in the absence and (b) in the presence of Zn(II) 400 p.p.b. in immobilized state.

Fig. 3. (a), (b) and (c) represent the relative enzyme activity of the immobilized alkaline phosphatase in the presence of inhibiting metal ions, Be(II), Zn(II) and Bi(III) respectively, against the activity in the absence of these metal ions.

3.2. Detection of Zn(II) by reactivation of immobilized apo-alkaline phosphatase

It was observed earlier in bulk solutions that apo-alkaline phosphatase exhibited reduced catalytic activity towards
CSPD as compared with the undialysed native enzyme [9]. The activity could be regenerated by adding Zn(II) ions in stoichiometric amounts. This fact was used to develop a sensitive chemiluminescence technique for the determination of Zn(II) ions using immobilized apo-alkaline phosphatase. Fig. 4 shows the calibration curve for Zn(II) ions by the regeneration of immobilized apo-alkaline phosphatase. A detection limit of 0.3 p.p.b. was achieved.

In an attempt to develop optical fibre-based biosensor applications for metal ion detection, the native enzyme was immobilized on the inner surface of a 100 μl glass capillary. Using identical concentrations of CSPD and the analyte to those used earlier, metal ions were detected to the same extent as in a test tube. Zn(II) was detected by both inhibition and activity generation (data not shown) methods. However, this protocol resulted in narrowed dynamic ranges due to surface area limitations.

Preliminary studies of enzyme immobilization on glass beads to develop a continuous flow cell were conducted. The glass beads with immobilized enzyme were subjected to 0.4 mM CSPD at a flow rate of 0.75 ml min⁻¹. This method yielded a raw signal two orders of magnitude greater than that observed for test tube-based immobilization. However, a lack of optimization of wash protocols for this method led to inefficient enzyme regeneration, adversely affecting the performance of the method. Currently investigations are under way to improve this methodology.

4. Conclusions

The metalloenzyme alkaline phosphatase was successfully immobilized by covalent cross-linking on a silane treated glass surface. Increased enzyme quantities on the surface were achieved by repetitive alternate treatment of the surface with glutaraldehyde and alkaline phosphatase. Three metal ion analytes which inhibit the enzyme were quantified. This system has been shown to detect Zn(II) down to 120 p.p.b., Be(II) down to 5 p.p.b. and Bi(III) down to 2.4 p.p.m. Reactivation of the immobilized apo-alkaline phosphatase allowed us to detect Zn(II) down to 0.3 p.p.b. A new method to detect trace levels of some metal ions has been demonstrated which forms the basis for an immobilized enzyme-based fibre optic sensor for metal ion detection. Further investigations are under way to build in selectivity to the detection system.

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