Study of Enzyme Kinetics using Capacitive Biosensor-An Alternate to Colorimeter

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Abstract
A novel sensor approach based on measurement of changes in capacitance of an electrochemical cell produced during enzyme catalyzed formation of products is described. The electrochemical property such as capacitance of an electrolyte between electrochemical cell depends on its ionic compositions, in turn depends on the resulting ionic constituents caused by catalytic action of the enzyme. Generation of micromolar (μM) concentration of ionic species causes large changes in capacitance that is measurable in picofarad (pF) range in capacitive electrodes. Broad clinical analytical application of this technique is demonstrated in this report by application to diagnostically valued enzymes measurements like pyrophosphatases and phosphatases. Specific measurements of enzyme activities were achieved over the concentration range of 0.125 – 5.0 mM of respective substrates. The performance of the electrochemical sensor based enzyme biochemical assays were compared directly with identical assays employing spectrophotometric detection and found to match well.

Keywords: Capacitance Changes, Electrochemical Cell, Enzyme Kinetics, Shigella Apyrase

1. Introduction
Enzyme, the molecular biocatalyst has been the subject of continued research for over three decades because of its universe significance. Determination of its concentration in aqueous sample is important in applied analytical chemistry and clinical sample analysis. Therefore, it is of great interest to devise novel assay methods of enzymes that are specific, fast and free from interference.

To date, there are many conventional methods, like spectrophotometric titrations being used to detect enzymes quantitatively, which normally involve using appropriate reagent after the enzymatic reaction. However; these methods have certain disadvantages like, requirement of unstable reagents, harsh conditions, expensive instrumentation and time consuming.

Many biochemical reactions involve changes in their charge status, a property that has not been adequately exploited for measuring enzyme activity. Today, there are much sensitive instrumentation for measuring changes and effects due to these charges such as capacitance. We have exploited this property and attempts were made to assay enzymes in this study. The main goal of this work is to develop instrumentation with electroporation cuvette as an electrochemical cell to measure the capacitance change on enzyme action and to circumvent the limitations of conventional methods and facilitating a more rapid return of clinical informations. The present work investigates the kinetic datas of clinically relevant enzymes like, apyrase, alkaline phosphatases with their substrates, Adenosine Triphosphate (ATP) and Para Nitro Phenyl Phosphate (pNPP) using capacitance measurements.

1.1 Enzymes Under Study
1.1.1 Apyrase
It is a class of calcium activated plasma membrane bound diphospho hydrolase enzyme that catalyses the reaction,

\[ \text{ATP} \rightarrow \text{ADP} \rightarrow \text{AMP} + 2\text{PO}_4^{3-} \]

This change in ion composition should result in change in the dielectric nature of the system and hence the capacitance of the system. Detection of Apyrase could be used as an indirect assay for the pathogen shigella spp., the causative agent of shigellosis, an infectious disease of the intestinal tract1.
1.1.2 Alkaline phosphatases (ALP)
These are a group of non specific enzymes, which hydrolyze organic phosphate esters liberating alcohol and inorganic phosphate. Urine from patient suffering from various diseases of kidney often contains high concentration of ALP and its detection could be an indirect method for detection of metabolic disorder, kidney diseases.

1.2 Electro Chemical Approach in Capacitive Sensor
In the system under study, the electrode-electrolyte interface is analogous to a capacitor. This is due to so called ‘double layer’ formed by excess of charges at the interface which may be ions, electrons or oriented dipoles. In capacitors, the introduction of dielectric results in increase in capacitance. Similarly in an electrochemical cell, the double layers at either metal electrode could act as a charged plate, the excess charge during enzymatic conversion of substrates in the metal surface over that in double layer contributing to it a net charge. This in turn analogous to a parallel plate capacitor with a highly dilute solution in between them acting as a dielectric. The capacitance that is being measured could be of parallel plate capacitor analogue.

2. Principle
Products formed during enzyme-catalyzed reactions modified the ionic composition of the electrolyte, which in turn resulted in changes in dielectric properties and capacitance of the electrochemical cell. Changes also occurred at electrode - electrolyte interface. Such modifications were proportional to the concentration of enzyme. The capacitance of the electrical double layer at an electrode can be calculated by the equation,

\[ C_{dl} = \varepsilon_0 \varepsilon_r A / d \]

\( \varepsilon_0 \) is equal to permittivity of free space, \( \varepsilon_r \) is dielectric constant of medium used, \( A \) is the surface area and \( d \) is the distance of the mobile charges to the electrode surface. By coupling changes in capacitance values to sensor format by using enzymes to catalyze the formation of products, it is envisaged that metabolic assays and immuno assays with extended dynamic ranges and improved sensitivities in comparison to other sensor formats may be produced.

3. Materials and Methods

3.1 Preparation of Apyrase
3.1.1 Bacterial Strain, Plasmid
E. coli GJ 1158 was used as the host strain. Plasmid, pRSETB was used as vector and the transformants were cultured aerobically at 37°C in LB medium without sodium chloride containing ampicillin.

3.1.2 Preparation of Periplasm
Recombinant E. coli GJ 1158 were grown in 3 ml LB medium to OD 590 0.6 and were then induced with 1M sodium chloride for 3 hours. 2 ml of culture was pelleted by centrifugation at 10,000 rpm for 5 minutes and suspended in 1 ml lysis buffer (20% Sucrose, 1mg/ml lysozyme, 50mMTris, 10mM EDTA) and again pelleted by spin at 10,000 rpm for 10 minutes and kept aside for 30 minutes and the supernatant was used as the periplasmic fraction.

3.2 Construction of Electrochemical Sensor
The electrochemical sensor consisted of an electrochemical cell of capacity 400 µl consisted of two aluminum electrodes separated by 2mm gap. Boonton capacitance meter with resolution of 1 pF was used as detector.

3.3 Capacitive Assay of Enzymes
The capacitive assay for apyrase involved the addition of enzyme to ATP and monitoring the change in capacitance of the mixture over reaction time. Apyrase (0.03µg/µl) activity was assayed in the presence of varying ATP concentrations (0.125-5 mM) in TE buffer. The extent of hydrolysis was determined by comparing the observed capacitance values with respective standard plot drawn for free phosphate molecule. For ALP (0.04 µg/µl) assay, the reaction procedure was the same as above, except that substrate used was pNPP and the obtained capacitance values was compared with standard graph plotted for p-nitrophenol(pNP).

One unit of an enzyme corresponds to the liberation of 1 µ mol of products min-1 mg-1 of protein.

3.4 Comparative Analysis to Capacitive Assay by Colorimetric Method.
Apyrase activity was measured according to method described by Bhargava, et al. In a micro plate assay format, 150 µl of assay volume contained 50mM Tris, 10mM EDTA, and 0.1-5mM ATP and the enzyme preparation. After
incubation for 15 minutes at 37°C, 100 µl of Chen’s reagent (2.5% ammonium molybdate, 6N sulphuric acid, 10% Ascorbic acid and water in the ratio of 1:1:1:2) was added. The principle of this assay is that, the phosphate released during apyrase action on ATP reacts with ammonium molybdate in color reagent forms phosphomolybdate complex which on reduction in acidic medium gives blue color that can be read at A₆₅₀. Previously a standard graph for phosphate was constructed using disodium hydrogen phosphate. Similarly colorimetric assay for ALP was also done with contained 0.1 M Na₂CO₃, 0.1 M NaHCO₃ buffer, pNPP and the enzyme preparation (0.2 µg/ml).50 µl of 1 N NaOH was added after reaction time and the OD was read at 414 nm and the values were compared with standard graph for various concentrations of pNP plotted under assay conditions.

4. Results and Discussion

The kinetic parameters (Vₘₐₓ, Kₘ and specific activity) were determined by varying concentrations of substrate [S] at constant enzyme concentration by using capacitive assay and typical Line weaver plot (LB) was obtained when the inverse of enzyme velocity was plotted against 1/[S] (Figure 4.1 and 4.2) using origin 7 and results were tabulated and compared with those obtained from colorimetric and literature values (Table 4.1).

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| Method         | Vₘₐₓ (µ mol min⁻¹) | Kₘ (mM) |
|----------------|--------------------|---------|
| Capacitive Assay |                   |         |
| Apyrase Alkaline | 25’10⁻³            | 1.11    |
| Phosphatases    | 14.3’10⁻³           | 1.25    |
| Colorimetric Assay |               |         |
| Apyrase         | 20’10⁻³             | 1.42    |
| Alkaline        |                    |         |
| Phosphatases    | 5.55’10⁻³           | 1.25    |
| Literature Value |                 |         |
| Apyrase         | 1.9 +/- 0.1         | 1 - 4   |
| Alkaline        |                    |         |
| Phosphatases    | 3.9 +/- 1.2         | 1 - 1.5 |
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This study suggests that the capacitive assay method exhibited similar kinetic datas which are comparable to those obtained by colorimetric method and matched well with literature values (Table 1) and is useful to assay for enzyme activity. On the other hand, the conventional colorimetric method presents some disadvantages since it is poor sensitive at low analyte concentration due to sharp and unequivocal color change of color reagent, time consuming and requires freshly prepared reagents. Also the cuvette which is used in this method proved to be an excellent electrochemical cell and provided cheapest mean for capacitive measurements.

5. Conclusions

The new electrochemical sensor using aluminum electrodes has been developed and fully characterized in this work. The feasibility of this electrochemical sensor to monitor the enzyme activity has been fully demonstrated. Work is in progress to use this approach to investigate the kinetic behavior of urease using different electrodes such as gold; silver to achieve sensitive detection of specific analytes. The measurement concept presented herein is generic since this type of sensing can be applied to any reactions which cause separation of ions thereby leading to capacitance changes.

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