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Enzyme Biosensor Electrode Based on Immobilized Urease-Alginate: Preparation, Characterization and Significance

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Abstract

Pigeonpea urease was immobilized on calcium alginate beads. The maximum immobilization, 50.5% was observed. The undertaken work compares the kinetic properties of soluble urease with the immobilized enzyme. The immobilized urease detected a shift in its optimum pH from 7.5 to 7.0 in Tris/acetate buffer. The optimim temperature also shifted from 47 to 67 degrees compared with the soluble enzyme. Alginate-immobilized pigeonpea urease had a higher K_m (10.5 mM) than that of the soluble enzyme (7.3 mM). An attempt has been made to use immobilized beads to prepare a urea biosensor developed from potentiometric pH glass electrode coupled to a calomel electrode. Furthermore, in a pilot study a calibration plot was made for estimation of serum urea using the enzyme biosensor electrode.

Keywords: Urease, immobilization, alginate, potentiometry.

Introduction

The enzyme urease (urea amidohydrolase, E C 3.5.1.5) is a nickel dependent metalloenzyme[1] and widely distributed in the nature.[2] The enzyme catalyzes the hydrolysis of urea to ammonia and carbon dioxide[3] as per the following chemical reaction: $NH_2CONH_2 + H_2O \rightarrow CO_2 + 2NH_3$.

The urease enzyme is biochemically important and plays a valuable role in determining levels of urea in the blood, urine and in waste water. Moreover, the enzyme has important medical applications, such as in the process of dialysis, where urea is removed from the blood in the treatment of uremia[4]. However, the full utilization of the enzyme's application could not be achieved due to several limitations. They include the enyzyme's high cost, unavailability, instability and poor recovery from a reaction mixture. On the other hand, the immobilized enzyme has been shown to be more stable and reusable, and, consequently, more economical. It should be pointed out that the immobilized enzyme generally displays lower catalytic activity than the free one,. Due to the several advantages, the development of new enzyme immobilization has created a tremendous excitement to the field and interest to many researchers[5-7]. Immobilization of urease has been performed in several matrices for various clinical/analytical applications[8], and has also been used for the treatment of urea-containing effluents[9]. For example, the assessment of blood urea has been successfully reported using several biosensors with immobilized ureases. One of the most notable methods employed is the entrapment of urease inside reversed micelles as a method of immobilization and the utilization of a glass electrode as a sensor [10,11] . In the present work, calcium alginate has been exploited for entrapment of enzymes (pigeon pea urease) as it is economical, stable and convenient to use. The present paper reports the study of urease immobilization on alginate beads. An attempt has also been made to estimate serum urea using enzyme electrode biosensor.

Material and Methods

Enzyme and chemicals: Urease (EC 3.5.1.5) was isolated from the seeds of pigeon-pea (*Cajanus cajan* L. Millsp.), which was purchased from the local market. The enzyme specific activity of the partially purified urease is 1850 - 2230 Units mg $^{-1}$ protein. Tris-buffer, urea and trichloroacetic acid were bought from Sisco Research Labs (Mumbai, India), Folin-Ciocalteau reagent and Nessler's reagent was purchased from Hi-Media (Mumbai, India). Bovine serum albumin (BSA), sodium alginate and Dialysis tubing were purchased from Sigma Chemical Co. (U.S.A.). Serum samples from the human subjects were collected from a local pathology laboratory (Varanasi, India). All reagents used in these experiments were of analytical grade. Unless stated otherwise, all reagents were prepared in double distilled water.

Immobilization of urease: 4% Na-alginate was prepared by dissolving 0.4 g of Na alginate in 10 ml of 50 mM Tris-acetate buffer (pH 7.3) at 40 ^{0}C; (b) 250 ml of 8% calcium chloride solution was freshly prepared and kept in ice bath.

Enzyme was mixed with 10 ml of Tris- buffer of Na–alginate (4%). The enzyme solution must be added at *ca* 0.1 mg ml $^{-1}$ alginate. This solution was taken in a syringe about 1 foot above the calcium chloride solution (8%) and allowed the alginate – enzyme mixture to fall drop wise from the syringe into a beaker containing 250 mL of chilled calcium chloride solution with constant shaking on a magnetic stirrer. As soon as the drop falls into calcium chloride solution, the free enzyme is gradually entrapped in a cage of calcium alginate in the shape of almost a bead. The number of beads formed was counted. After the

formation of beads, the solution was left for stirring for 2 hours. Beads were collected and washed thoroughly with the buffer and stored suspended in buffer at 4°C.

Enzyme activity assay: For the measurement of soluble urease activity, the amount of ammonia generated during a certain time period at a saturating concentration of urea was determined [12]. To initiate the reaction 0.2 M urea was added to the enzyme, which was suitably diluted in 50 mM Tris-acetate buffer (pH 7.3) following incubation for 10 min. The reaction was stopped by the addition of 10% (w/v) trichloroacetic acid (TCA). The colour was developed in the supernatant using Nessler's reagent. The yellow-orange colour, thus generated, was measured colorimetrically at 405 nm wave length. Then the amount of NH_3 liberated in the test solution was calculated by calibrating the reagent with standard NH_4Cl solution. The definition of the enzyme used here is the amount required to produce 1 micro mol of ammonia/min under our test conditions [0.05 M Tris/acetate buffer (pH 7.3) containing 0.2 M urea at 37^0 C.

To assay immobilized enzyme, the beads were incubated in 0.05 M Tris/acetate buffer (pH 6.5) containing 0.2M urea in different test tubes at 37^0C with intermittent shaking. Following incubation for the desired time, an aliquot (1ml) was taken out from the reaction mixture and assayed as described above[12]. The percentage of immobilization is defined as the ratio of (total activity in immobilized beads / total activity of the soluble enzyme loaded) x 100.

Protein concentration was determined as per the standard method of Lowry et al[13] using the Folin–Ciocalteu reagent, and BSA as the protein standard. The amount of protein immobilized was calculated by subtracting the amount of protein in the supernatant fraction following immobilization from the total amount of protein used for the immobilization. In our hands, the percentage immobilization was found to be about 50.5.

Steady state kinetics: The optimum pH for the activity of the immobilized urease was calculated by varying the pH of the assay buffer, which is 0.05M Tris/acetate, ranging from 5.0 to 9.0. The enzymatic activity was determined for each buffer by the method described for the enzyme assay above. For each pH value tested, fresh beads were used. All further studies were performed at the optimum pH. The Km of the immobilized urease was determined by varying the urea concentration from 5 to 200 mM and constructing a Lineweaver-Burk plot. Optimal temperature was tested by varying the temperature of immobilized urease; the activity of immobilized urease was assayed at increasing temperatures ranging from 27 ^{0}C to 77 ^{0}C.

Construction of a urea biosensor and assay of urea in serum: Approximately10–12 immobilized beads were kept in a dialysis tube (4.5 cm*1.5 cm). The bottom of the tubing was tied with the help of a thread. A glass pH electrode was inserted from the open end of the tubing and this end of the dialysis tubing was then tied above the electrode bulb. The optimal buffer [10 ml of 0.025 M Tris-acetate buffer (pH 6.5)] was first placed in a beaker, which was then shaken at a moderate speed using a magnetic stirrer. The electrode was immersed in the solution. When the electrode potential across the two leads of the composite glass electrode (glass electrode coupled to a calomel electrode, using a Elico L1 127 pH meter) attained a stable value, 500 *ul* of a urea solution of known concentration was added. The stable value reached was recorded. Various concentrations of urea were used so that a calibration curve could be plotted.

To measure the urea content of blood serum, 10 ml of 0.025 mM Tris/acetate buffer (pH 6.5) was placed in a beaker. Serum (500 *ul*) was added and the electrode potential was measured as described above. Urea concentration was subsequently determined by the calibration plot.

Results and Discussion

The fabrication of a biosensor generally involves different steps, and the enzyme immobilization step is found to be one of the most crucial steps. The advantage of enzyme immobilization is significant. Immobilized enzymes display greater stability and higher activity than soluble enzymes. The present study has two major aims. The first aim was to immobilize urease by entrapping with the help of Na-alginate and calcium chloride. The second aim was to determine the degree of enhancement in the activity of the entrapped enzyme.

Effect of pH on immobilized enzyme: The effect of pH on the activity of free and immobilized urease is presented in the Figure-1. As per the data, the pH optima of the soluble and immobilized urease were found to be 7.5 and 7.0 respectively in 0.05 M Tris /acetate buffer. In the case of immobilization of pigeon pea urease, a shift was observed from pH 7.5 (soluble) to pH 7.0 (immobilized) in 0.5 M Tris acetate buffer. However, in the case of chitosan immobilization of pigeon pea urease, a shift towards basic pH value[14] was detected from 7.3 (soluble) to 8.5 (immobilized) in 0.05 M Tris-acetate buffer.

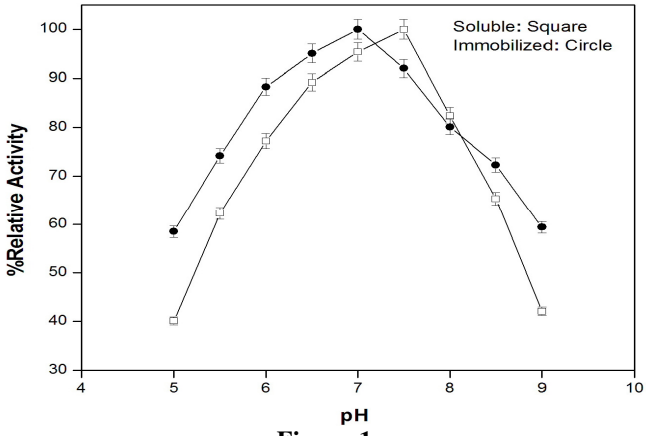

Figure-1
Effect of pH on soluble and alginate -immobilized pigeon pea urease

The behaviour of an enzyme can be subjected to change by its immediate micro-environment. For instance, an enzyme can have an altered pH optimum upon immobilization on a solid matrix in relation to its pH optimum in solution. Likewise, the pH value in the immediate vicinity of the enzyme molecule may change, which depends on the surface and residual charges on the solid matrix and the nature of the enzyme bound. Therefore, these interactions are expected to cause a shift in the pH optimum of the enzyme[15=17].

Effect of temperature on immobiiized enzyme: Results of the effect of temperature on soluble and immobilized urease are presented in the figure-2. As per the results, soluble urease from pigeon pea has an optimum temperature of 47^0C whereas for alginate entrapped urease was stable up to 67^0C.

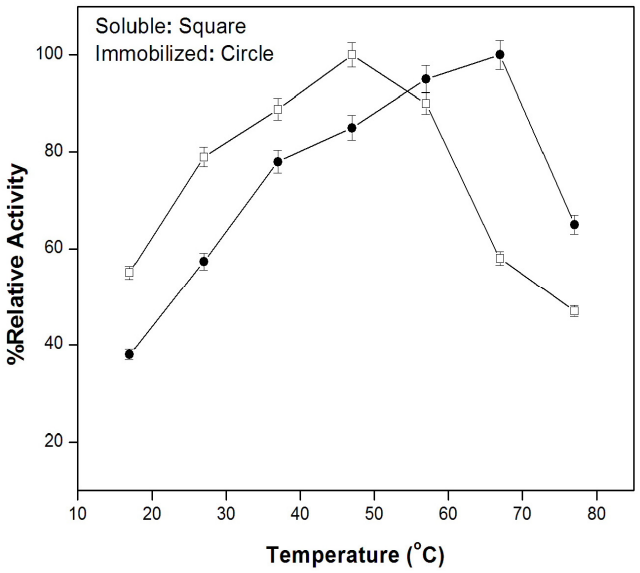

Figure-2
Effect of temperature on soluble and alginate-immobilized pigeon pea urease

A significant increase in the optimum temperature was noticed when urease was entrapped with alginate. It is worthwhile to state that under normal conditions of activity in living cells most of the enzymes exist in a membrane bound form or bound to other macromolecules and are not usually found in the free state as in *in vitro* state [15-17] .

Effect on Km: Urease immobilized showed an apparent Km value of 10.5 mM which is higher than the Km of the soluble enzyme 7.3 mM (data not shown). This is an interesting observation, which is believed to result from the presence of an unstirred layer of solvent that surrounds suspended water insoluble particles. This unstirred layer is termed as the Nernst layer. Indeed, a concentration gradient of substrate is established across the layer with water insoluble enzymes i.e. immobilized enzymes. Therefore, the saturation of an enzyme attached to a water insoluble particle would result at a higher substrate concentration than normally required for the saturation of the freely soluble enzyme. The net results would be an increase in the Km value as observed herein..

Assay of urea in serum using the biosensor: The biosensor (described under the Materials and Methods section) prepared from the alginate beads (10–12) was used for estimation of serum urea samples. Levels of urea were assayed in five serum samples (clinical samples obtained from the local pathology laboratory) within 30 minutes. The normal physiological range of urea is 20-45 mg/dl. The result of urea assay from serum samples is summarized in table-1. The calibration curve generated by plotting the potential difference (mV) across the two leads of the combined glass electrode against the concentration of urea is presented in figure-3. Urea content in five clinical samples was found to be in the range: 21 -50 mg/dl.; this range reflected the concentrations of urea that are common in clinical samples.

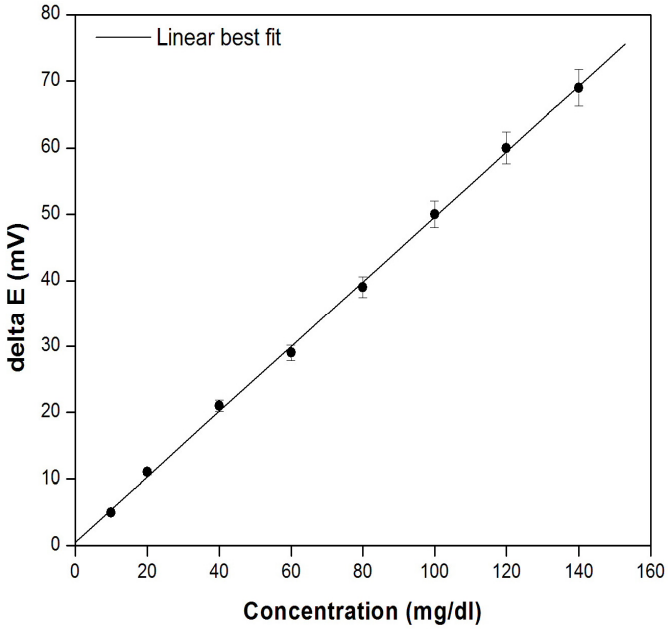

Figure-3
Calibration Plot for estimation of serum urea using enzyme biosensor electrode

Table-1
Determination of serum urea content using biosensor

Sample number	Urea (mg/dl)
1	21.2
2	25.0
3	33.4
4	50.4
5	32.0

The normal physiological range: 20-40 mg/dL

To confirm the validity of the calibration curve, the concentration of a few samples of known strengths (30 and 90 mg of urea/dl) within the range of the detection limits, but not utilized for constructing the calibration curve, were determined by using the present device. Further studies are needed to ascertain the optimum performance of the electrode in terms of reproducibility, response time and reliability of the biosensor.

Conclusion

Calcium alginate was an adequate and useful material for urease immobilization to prepare the potentiometric urea biosensor. Estimation of serum urea using enzyme biosensor electrode was successfully performed. In particular, more prospective studies and clinical trials are necessary to determine the efficiency and utility of the biosensor constructed by us. Notably, enzyme biosensor electrode in the present study for estimation of urea in serum samples may be an alternative substitute of autoanalyzer.

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