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Urease immobilized polymer hydrogel: Long-term stability and enhancement of enzymatic activity

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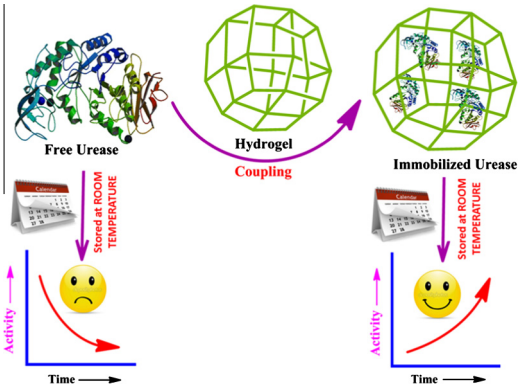
GRAPHICAL ABSTRACT

A novel method using polymer hydrogel based material has been developed for long-term stabilization of enzymes in room temperature and increase in catalytic activity with storage duration.



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ABSTRACT

A method has been developed in which an enzyme namely urease was immobilized inside hydrogel matrix to study the stability and enzymatic activity in room temperature (~27–30 °C). This urease coupled hydrogel (UCG) was obtained by amine-acid coupling reaction and this procedure is such that it ensured the wider opening of mobile flap of enzyme active site. A systematic comparison of urea–urease assay and the detailed kinetic data clearly revealed that the urease shows activity for more than a month when stored at ~27–30 °C in case of UCG whereas it becomes inactive in case of free urease (enzyme in buffer solution). The aqueous microenvironment inside the hydrogel, unusual morphological features and thermal behaviour were believed to be the reasons for unexpected behaviour. UCG displayed enzyme activity at basic pH and up to 60 °C. UCG showed significant enhancement in activity against thermal degradation compared to free urease. In summary, this method is a suitable process to stabilize the biomacromolecules in standard room temperature for many practical uses.

1. Introduction

Enzymes are being used in numerous areas including biomedical and biotechnological applications. Protein/enzyme based

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medicines have attracted much interests in biomedical industry as they can control and cure diseases [1]. Often sub-ambient temperature is required for storing the proteins/enzymes as they can sustain only for limited time in absence of native condition. Hence protein/enzyme stability in room temperature (~27–30 °C) is still a challenge for protein/enzyme based medicines and therapeutics. Therefore, it is interesting to develop methods which can preserve proteins/enzymes biological activity in room temperature and hence a significant amount of research is required to address this issue [2–4]. A large numbers of proteins, enzymes and DNA have been reported to retain catalytic activity in ionic liquid medium [2–8] particularly lipases maintain their activity in anhydrous, hydrophobic ionic liquids. Their selectivity and operational stability are sometimes even better than in traditional volatile solvents [2–8].

Immobilization of enzyme on to a substrate increasingly becoming important compared to free enzyme (means enzyme used as solution) for all practical purposes because former offers many operating and handling advantages such as can be utilized in either batch or continuous processes, low reaction times for product formation, high yields of product, increase in stability, reduction in the operating cost and also easy recovery of the enzyme [9–13]. Numerous examples of immobilized enzymes have been discussed in the literature for their uses in analysis, industrial process, biotechnology, biomedical and bioengineering applications [14,15]. Varieties of immobilization techniques have been developed in recent years and the solid supports were chosen based on the type of applications [16,17].

Immobilization of enzymes on soft and solid supports like hydrogels is an effective process among the various enzyme immobilization methods. Since hydrogel, especially polymer hydrogels which are cross linked three-dimensional network arrangement of polymers, retains large amount of water inside the three dimensional network and hence it provides a suitable physiological conditions. The aqueous environment of polymer hydrogel can minimize the denaturation of enzymes and help to carry out the enzymatic functions. Therefore, one can expect to retain the enzyme activity upon immobilization into the polymer hydrogel matrix. Among the various polymer hydrogels poly(ethylene glycol) (PEG), poly(2-hydroxylethylmethacryloate) (PHEMA), poly(N-isopropyl acrylamide) (PNIPAM) and acrylamide copolymer based hydrogels are the most often used materials for several enzymes immobilization [18]. Acrylamide based copolymer hydrogels are very convenient for enzyme immobilization because of its hydrophilic nature, resistance towards microbial or enzymatic attacks, high chemical and mechanical stability [19,20].

Enzyme urease catalyses the hydrolysis of urea to unstable carbonic acid, which on immediate decomposition produce ammonia and carbon dioxide [21]. Immobilized urease used for the construction of artificial organ machines, biosensors [22,23] for the quantification of urea and Hg^{2+} form aqueous solutions analytically [24], bioreactors for dialysis regeneration systems in artificial kidney machines [25], in food industry for removing urea from foods and beverages [26]. Immobilization of urease on numerous materials has been reported by several groups [23,27–30].

Like most of the other enzymes, urease also display very poor stability and complete loss of enzymatic activity in room temperature since the native protein structure undergoes into deactivated state. Therefore, development of a simple and robust method for long term stability of urease enzymes in room temperature is a challenging task and successful implementation will have immense effect on the widespread use of urease. Although several attempts have been made in the past, however, most often either methods are quite complex or enzymatic activity gradually decreases with storage time. Hence, the current challenge in this area is to develop a method by which urease can be stored in the temperature range ~27–30 °C for long time without losing its biological efficiency.

In this article, we have developed enzyme immobilized polymer hydrogel to address these key issues. We have used urease as a model enzyme because of its cheap commercial availability, easy assay method and interesting class of structure. Urease is covalently immobilized onto poly (acrylamide-co-bisacrylamibe) hydrogel and urease–urea hydrolysis kinetics in aqueous media is used as assay to access stability for long time in room temperature. The physicochemical properties, the operational stability in room temperature with storage time are discussed in detail by comparing free urease in solution and immobilized (in hydrogel) urease. We believe similar studies can be carried out for other enzymes as well.

2. Experimental section

2.1. Materials

Urease (Jack bean Conavalia ensiformis, MW 480 KDa) with specific activity of 57U/mg was purchased from Sigma Aldrich and used as received, Acrylamide (AA), N,N′-methylenebisacrylamide (BAA), diethoxyacetophenone (DEAP), *N,N,N′,N′*-tetramethylethylenediamine (TEMED), ethyl-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and urea were purchased from Sigma Aldrich and used as received. Dimethyl sulfoxide (DMSO, Fisher), NaOH, phenol, sodium nitroprusside, sodium hypochlorite (NaOCl) (contains 5% active chlorine) were purchased from Merck, India and used as received. Water (HPLC grade) was purchased from Fisher Scientific and used to make all aqueous solution. 100 mM phosphate buffer saline (PBS) with 150 mM NaCl in it was used as a buffer throughout the studies unless otherwise specified. Two reagents (Reagent A and B) for the assay of urease–urea reaction were prepared as follows: Reagent A was prepared by dissolving phenol (5 g, 53 mmol) and sodium nitroprusside (25 mg, 0.084 mmol) in 500 ml of water, while Reagent B was prepared by dissolving NaOH (2.5 g, 0.0625 mmol) and NaOCl which contains 5% active chlorine (4.2 mL) in 500 mL of water.

2.2. Methods

2.2.1. Preparation of hydrogel

AA (0.100 g, 1.40 mmol), BAA (0.01 g, 0.03902 mmol), 2.00 mL of water were mixed together using a vortex mixer in a 10 ml screw cap glass vial, then 4–5 drops of 10% v/v DEAP (7.7 μL, 3.84 mol) in DMSO was added into the above reaction mixture and mixed thoroughly using vertex mixer. This solution was nitrogen bubbled for five minutes to remove any dissolved oxygen. The polymerization mixture was then injected into a polymerization cell (size: 3 cm × 3 cm) consisting of two quartz disks, separated by a 127 μm thick parafilm spacer with the help of disposable syringe. The polymerization cell was placed under mercury lamp (Black Ray) operating at a wavelength of 365 nm for 4 h. After the completion of the reaction, the quartz cell was opened in HPLC grade water followed by thoroughly washing of obtained hydrogel with HPLC grade water for several times to remove the unreacted monomers and extra initiator. Then the hydrogel was stored into the water for further use.

2.2.2. Characterization/analysis/instrumentation

UV–Vis spectroscopic analysis was carried out in absorbance mode on a carry 100 Bio UV–Vis spectrophotometer to study the urease–urea hydrolysis kinetics. Mercury Lamp (Black Ray, operating at 365 nm) was used for photo-polymerization. Thermogravimetric and differential thermal analysis (TG-DTA) were carried