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Polyethylene terephthalate membrane as a support for covalent immobilization of uricase and its application in serum urate determination

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ABSTRACT

A method is described for covalent immobilization of uricase onto polyethylene terephthalate (PET) membrane with a conjugation yield of 4.44 μg/cm^2 and 66.6% retention of initial activity of free enzyme. The enzyme exhibited an increase in optimum pH from pH 7.0 to 8.5 and K_m for uric acid from 0.075 mM to 0.13 mM but slight decrease in temp. for maximum activity from 37 °C to 35 °C after immobilization. A colorimetric method for determination of serum uric acid was developed using immobilized uricase, which is based on measurement of H_2O_2 by a color reaction consisting of 3,5-dichlorobenzene sulphonic acid (DHBS), 4-aminoantipyrine and peroxidase as chromogenic system. Minimum detection limit of the method was 0.05 mM. Analytical recovery of added uric acid (5 mg/dl and 10 mg/dl) was 94.3% and 89.8%, respectively. Within and between batch coefficient of variation (CV) were <3.2% and <4.3%, respectively. A good correlation (r = 0.98) was found between uric acid values by standard enzymic colorimetric method and the present method. The immobilized uricase was reused 100 times during the span of 60 days without any considerable loss of activity, when stored in reaction buffer at 4 °C. The support chosen for the present study was biocompatible, antimicrobial, inert, impact resistant, light weight and had good shelf life.

1. Introduction

Uric acid (UA) is the principal end product of purine metabolism in human [1] hence, its determination serves as a marker for the detection of disorders associated with purine metabolism such as gout and Lesch–Nyhan syndrome [2]. Hyperuricemia is an indicator of kidney injury [3], leukemia [4] and pneumonia [5]. Uric acid is also associated with hypertension, hyperlipidemia and glucose intolerance, which play causal role in cardiovascular diseases [6]. The monitoring of serum UA levels is therefore essential in the follow-up of hyperuricemic patients to give them adequate feedback and treatment guidelines. Methods based on uricase and mass fragmentography have been proposed as reference methods. However, mass fragmentography is not suitable for routine clinical practice and offers only a certified reference method for calibration purpose [7]. In other techniques such as electrochemical [8] HPLC [9,10] chemiluminescence [11] and fluorescence methods [12] either substances coexisting with uric acid can affect the determination or the related sample preparation procedure is time consuming and certain amounts of uric acid may also get oxidized [13]. Nevertheless enzymic colorimetric/spectrophotometric methods are much more popular in clinical practice, since the enzyme had a unique ability of molecular recognition [14,15]. However, the enzymes are expensive. Immobilization of the enzymes onto insoluble supports not only permits their easy separation of the reaction medium but also, if properly performed, may improve their activities, stability and selectivity (e.g., if multipoint or multisubunit immobilization is obtained) [16–18] and often reduces the cost of procedure [15]. Uricase has been immobilized onto various supports such as dextran and polyethylene glycol [19] nylon tubing [20], cellulose acetate membrane [21]. The discrete analysis of uric acid in serum and urine employing alkylamine glass bead bound uricase and peroxidase have been reported from this lab [15,22]. Though the alkyl/aryl amine glass beads/matrix are generally resistant to microbial attack, yet the beads are expensive. Like wise some of these supports have poor shelf life and less stability. In view of the stupendous properties associated with polyethylene terephthalate (PET) membrane such as biocompatible, antimicrobial nature, inert, impact resistant and light weight [23], PET membrane was chosen as a potential support for immobilization of uricase for its application in serum uric acid determination. Recombinant uricase used herein is a tetrameric protein with an optimum pH of 7.0. It is neither glycosylated nor requires any cofactor for its activity [24]. At pH 7.0, –NH_2 groups of lys on the surface of this enzyme have very low activity, because of low pKa value and therefore only –NH_2 terminal groups of enzyme might be expected to react during immobilization. In the present study, alkali and acid treatment of PET membrane is expected to generate –COOH groups on PET

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membrane, which react with $-NH_2$ terminal group of each subunit of enzyme through carbodiimide linkage and thus provides a multipoint immobilization.

2. Experimental

2.1. Materials and methods

Chemicals: Recombinant uricase from Arthrobacter globiiformis (>2 U/mg), glutaraldehyde (25%), bovine serum albumin (BSA), 4-aminophenazone and 3,5-dichloro-2-hydroxy benzene sulphonic acid (DHBS) were from Sigma, USA. Horseradish peroxidase (HRP) was from SRL (Mumbai). All other chemicals were used of AR grade. Polyethylene terephthalate (PET) membrane (1 × 1.5) was purchased from local market.

2.1.1. Dissolution of uricase

Commercial recombinant uricase (1 mg) was dissolved in 1 ml 0.05 M glycine–NaOH buffer, pH 8.8 and stored at 4 °C until use.

2.1.2. Preparation of color reagent

It was prepared as described by [22] with modification. The color reagent consisted of 61 mg 4-aminophenazone, 1.035 g 3,5-dichloro-2-hydroxybenzene sulphonic acid (DHBS) and 300 units of horseradish peroxidase in 100 ml of 0.4 M sodium phosphate buffer (pH 7.0). It was stored in brown colored bottle at 4 °C and prepared fresh every week.

2.1.3. Assay of free uricase

Assay of uricase was based on the measurement of H_2O_2 generated in the uricase reaction by a color reaction with 4-aminophenazone, 3,5-dichloro-2-hydroxybenzene sulphonic acid (DHBS) and horse radish peroxidase as a chromogenic system [19]. The enzyme assay was carried out in a 15 ml test tube wrapped with black paper. Assay mixture contained 1.8 ml of 0.05 M glycine–NaOH buffer (pH 8.8), 0.1 ml of uric acid (1 mM) and 0.1 ml of uricase enzyme (0.2 unit/ml). The blank tube contained 1.9 ml of glycine NaOH buffer pH 8.8 and 0.1 ml of uric acid (1 mM). After incubation at 37 °C for 5 min, 1.0 ml color reagent was added to reaction mixture and kept at room temperature for 15 min in dark to develop the color A_{520} was read in Spectronic-20D and content of H_2O_2 was extrapolated from standard curve of H_2O_2.

2.1.4. Unit of enzyme

One unit of enzyme is defined as the amount of enzyme which generate 1 μmol of H_2O_2 per min under standard assay conditions.

2.2. Immobilization of uricase on PET membrane

2.2.1. Activation of PET membrane

The pieces of 1 cm × 1.5 cm size were cut from PET membrane and dipped into 10 ml 10 M NaOH overnight. Next day, the pieces were taken off with a forcep and placed into 10 ml 50% H_2SO_4 for 2 h. The pieces were then washed repeatedly with 0.05 M glycine–NaOH buffer, pH 8.8. The uricase (0.5 ml) was poured onto activated PET pieces for immobilization [25]. The unbound enzyme was removed from PET pieces with eppendroff pipette and tested for activity and protein content.

2.3. Assay of immobilized enzyme

Assay of PET membrane bound uricase was carried out as described for free uricase except that dissolved uricase was replaced by PET membrane bound uricase and the reaction buffer was increased by 0.1 ml and kept under continuous stirring during the assay. After the completion of the assay, the reaction mixture was taken off the test tube and membrane was washed in the reaction buffer for 3–4 times to remove color reaction residues.

2.4. Kinetic properties of immobilized uricase

To validate the conditions of optimum pH, temperature, incubation period and substrate concentration on immobilized enzyme, the pH of reaction buffer was varied from pH 7.0 to 10.0 at an interval of pH 0.5 using the 0.05 M glycine–NaOH buffer, the incubation temperature from 20 °C to 45 °C at a interval of 5 °C, the time of incubation was from 10 min to 30 min at an interval of 5 min and the concentration of uric acid was varied from 0.1 mM to 0.7 mM. K_m and V_{max} were calculated from Lineweaver Burk plot.

2.5. Determination of serum uric acid

Serum samples (1 ml each) from the 20 apparently healthy persons were collected from the local Pt. BDS PGIMS hospital. Uric acid content was determined in these serum samples by PET pieces bound uricase in the same manner as described for assay of immobilized enzyme, under optimized working conditions except that uric acid solution was replaced by serum. The concentration of uric acid in serum sample was extrapolated from the standard curve of uric acid. The method was evaluated by studying analytical recovery, precision and correlation with standard enzymic colorimetric method employing free uricase.

2.6. Reuse of immobilized enzyme

To reuse the immobilized enzyme, the PET pieces bound uricase was washed by dipping it in series of 3–4 test tubes containing 2 ml of reaction buffer. The immobilized PET strip was stored in reaction buffer at 4 °C when not in use.

3. Results and discussion

3.1. Immobilization of uricase on PET membrane

Commercial recombinant uricase has been immobilized on polyethylene terephthalate (PET) membrane through carbodiimide linkage with 66.6% retention of initial activity of free enzyme and a conjugation yield of 4.44 μg/cm^2 (Table 1). Polyethylene terephthalate is a thermoplastic polymer resin of the polyester family. The strong alkali and acid treatment, i.e. acid base hydrolysis of PET pieces generated –COOH groups on its surface. The activated PET pieces with protruding –COOH groups reacted with $-NH_2$ groups on the enzyme surface to form carbodiimide linkage [26] (Fig. 1).

The carbodiimide linkage has advantage over other types of covalent linkages, which involve schiff base formation due to glutaraldehyde and HNO_2 (diazotization reaction) that it does not require any coupling agent and the resulting linkage is much stronger than the above mentioned ones.

3.2. Kinetic properties of immobilized enzyme

A comparison of various kinetic properties of PET membrane bound uricase is given in Table 2. The immobilized enzyme showed maximum activity at pH 8.5, which is higher than that of free enzyme 7.0 [27]. The change in optimum pH of enzyme after immobilization might be due to many reasons such as an improved stability at that pH, the prevention of dissociation, a internal pH different from the external pH and even a chemical modification of the enzyme. The optimum pH of polyethylene terephthalate sheet bound uricase is slightly lower than those immobilized on other supports like free alkylamine glass (pH 8.8) [15]; free arylamine glass (pH 8.8) [28]; and affixed alkylamine glass (pH 8.8) [29]. The

Table 1
Immobilization of commercial uricase onto polyethylene terephthalate strip.

Uricase added to membrane (mg)	Uricase coupled to membrane (mg)	Total activity[a] added (units)	Total activity bound (units)	% retention of activity	Conjugation yield (μg/cm^2)
0.166	0.095	18	12	66.6%	4.44

[a] One unit of uricase is equivalent to 1 nmol H_2O_2/min activity of the enzyme.

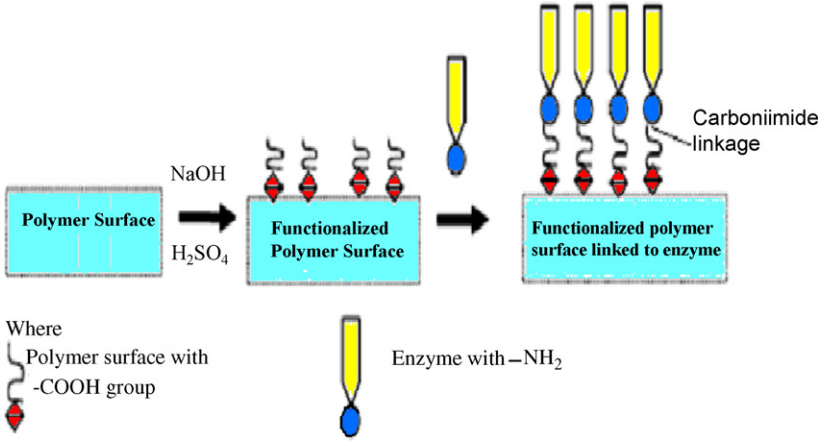

Fig. 1. Mechanism of covalent immobilization of enzyme (uricase) on polyethylene terephthalate membrane.

Table 2
A comparison of kinetic parameters of uricase immobilized onto polyethylene terephthalate strip with those reported on other supports.

Properties	Bhargava et al. [15]	Bhargava and Pundir [28]	Sushma et al. [29]	Present method
Source of uricase	Porcine liver uricase	Porcine liver uricase	Recombinant bacterial uricase	Recombinant enzyme from Sigma
Support for immobilization	Alkylamine glass beads	Free alkylamine glass beads	Alkylamine glass beads affixed on a plastic strip	Polyethylene terephthalate strip
Method of immobilization	Glutaraldehyde crosslinking	Glutaraldehyde crosslinking	Glutaraldehyde crosslinking	Glutaraldehyde crosslinking
Optimum pH	8.8	8.8	8.8	8.5
Optimum temperature	40 °C	40 °C	40 °C	35 °C
V_{max}	*Not reported*	*Not reported*	0.068 μmoles H_2O_2/min	18.867 nM/min
K_m	*Not reported*	0.13 mM	0.15 mM	0.13 mM

immobilized enzyme had optimum temperature of 35 °C, which is slightly lower than that of free enzyme (37 °C) [27]. The optimum temp. of PET membrane bound enzyme is lower than those reported on other supports like free alkylamine glass (40 °C) [15], and affixed alkylamine glass (40 °C) [29]. The rate of reaction for immobilized enzyme was linear up to 20 min after which it was constant and hence in all subsequent assays the reaction mixture was incubated for 20 min. This incubation time is slightly lower than that immobilized on alkylamine glass (25 min) [15]. K_m for uric acid was 0.13 mM, which is higher than that reported for free enzyme (0.075 mM) [22] but almost similar to that immobilized onto free and affixed alkylamine glass (0.13 mM and 0.15 mM) [15,29]. The change in the kinetic properties of enzyme after immobilization might be due to change in enzyme conformation, steric effects, micro environmental effects, and bulk and diffusion effects [30]. The following analytical parameters were determined to evaluate the method.

3.3. Serum urate determination with immobilized urate

A colorimetric method was developed for determination of urate using immobilized uricase, respectively. The method is based on measurement of H_2O_2 by a color reaction using DNBS, 4-aminophenazone and HRP as chromogenic system. The method allows the reuse of immobilized enzyme with ease. The support used for immobilization of enzyme is totally inert, light weight impact resistant and firmly attached with enzyme.

3.3.1. Linearity

Immobilized uricase showed a linear relationship between its activity and uric acid concentration in the range, from 0.05 mM to 0.5 mM (Fig. 2), which is in normal metabolic range (0.20–0.47 mM). Hence the clinical samples containing uric acid

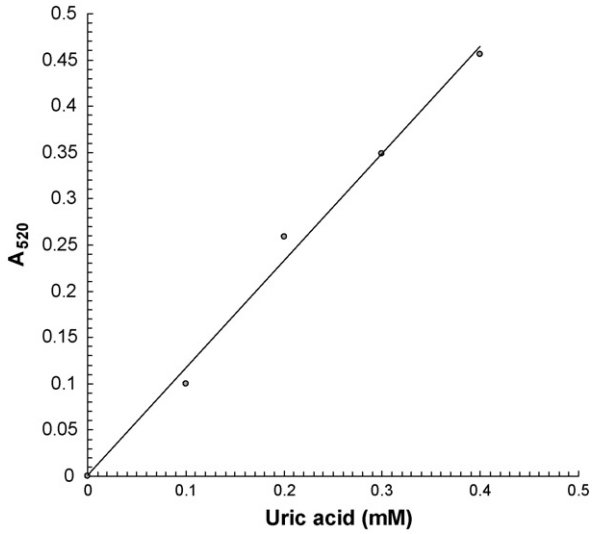

Fig. 2. Standard plot of uric acid by polyethylene terephthalate bound uricase.

Table 3
Analytical recovery of added uric acid in serum by immobilized uricase onto PET strip.

Uric acid added (mg/dl)	Uric acid found (mg/dl)	% Recovery
–	5.8	–
50	8.7	94.3
100	9.8	89.8

Table 4
Within and between assay coefficients of variation (CV) for determination of serum uric acid by immobilized uricase onto PET strip.

(*n*)	Mean uric acid (mg/dl)	CV (%)
Within assay (6)		
4.32	4.65	3.2 ± 0.15
5.12		
4.45		
5.09		
4.32		
4.62		
Between assay (6)		
3.38		
3.52	3.22	4.3 ± 0.14
2.99		
2.41		
3.52		
3.51		

level higher than metabolic level, should be diluted suitably before their analysis by the present method.

3.3.2. Detection limit

The minimum detection limit of this method was 0.05 mM [27,28].

3.3.3. Recovery

The analytic recovery of added uric acid in serum sample, i.e. 5 mg/dl and 10 mg/dl was 94.3 ± 2.34% and 89.8 ± 3.17%, respectively, which is comparable to earlier reports (Table 3).

3.3.4. Precision

To study the reproducibility and reliability of the present method, the uric acid content in same serum sample was determined six times in a day (within batch) and these serum samples were analyzed similarly after their 1 week storage at −20 °C (between batch). The within and between batch CVs were <3.2% and <4.3%, respectively (Table 4).

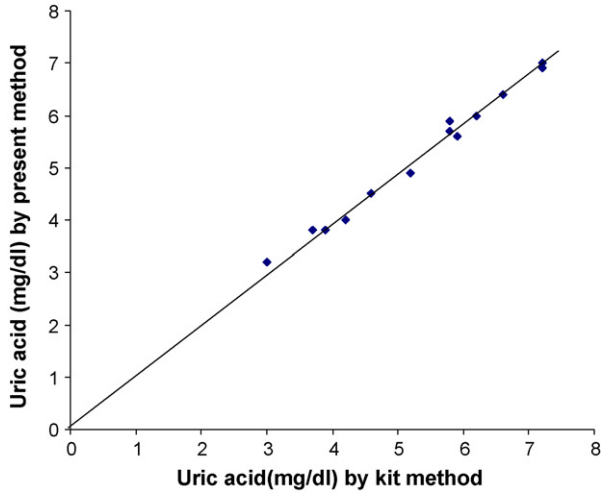

Fig. 3. Correlation between serum uric acid values determined by standard enzymic colorimetric method (*x*) and PET membrane bound uricase (*y*).

3.3.5. Accuracy

To test the accuracy of method, uric acid value in serum obtained by present method (*y*) was compared with those obtained by standard enzymic colorimetric method using free enzyme (*x*). The serum uric acid values obtained by both the methods showed a good correlation ($r = 0.98$) giving the following regression equation $y = 0.9239x + 0.2826$ (Fig. 3).

3.3.6. Serum urate determination

The uric acid content in serum as measured by the present method was in the range 0.20–0.36 mM with a mean of 0.29 mM in apparently healthy males and 0.14–0.306 mM with a mean of 0.85 mM in apparently healthy females. These values are in normal accepted range of 0.20–0.47 mM for serum uric acid.

3.3.7. Interference studies

Among the various metal salts and metabolites tested such as NaCl, $MgSO_4$ and $CaCl_2$, glucose, cholesterol, urea, ascorbic acid at their physiological conc., only NaCl and glucose showed 11% and 12% stimulation in the activity of immobilized enzyme.

3.3.8. Reusability and storage stability

The enzyme electrode showed only 32% loss of initial activity after its 100 uses over a period of 60 days when stored in reaction buffer at 4 °C.

4. Conclusion

Commercial uricase was immobilized onto polyethylene terephthalate (PET) membrane through carbodiamide linkage membrane, optimized and used in determination of uric acid level in serum. It exhibits good stability with a shelf life of at least 2 months. The present work indicated that polyethylene terephthalate (PET) membrane material is a promising support for multisubunit immobilization for multimeric enzymes [31].

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