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Fabrication of dissolved O_2 metric uric acid biosensor using uricase epoxy resin biocomposite membrane

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ABSTRACT

Uricase purified from 20-day-old leaves of cowpea was immobilized on to epoxy resin membrane with 80% retention of initial activity of free enzyme and a conjugation yield of 0.056 mg/cm^2. The uricase epoxy resin bioconjugate membrane was mounted over the sensing part of the combined electrode of 'Aqualytic' dissolved O_2 (DO) meter to construct a uric acid biosensor. The biosensor measures the depletion of dissolved O_2 during the oxidation of uric acid by immobilized uricase, which is directly proportional to uric acid concentration. The biosensor showed optimum response within 10–12 s at a pH 8.5 and 35 °C. A linear relationship was found between uric acid concentration from 0.025 to 0.1 mM and O_2 (mg/l) consumed. The biosensor was employed for measurement of uric acid in serum. The mean value of uric acid in serum was 4.92 mg/dl in apparently healthy males and 3.11 mg/dl in apparently healthy females. The mean analytic recoveries of added uric acid in reaction mixture (8.9 and 9.8 mg/dl) were 93.6 ± 2.34 and 87.18 ± 3.17% respectively. The within and between batch CVs were <6.5 and <5.0%, respectively. The serum uric acid values obtained by present method and standard enzymic colorimetric method, showed a good correlation (r = 0.996) and regression equation being $y = 0.984x + 0.0674$. Among the various metabolites tested only, glucose (11%), urea (38%), NaCl (25%) and cholesterol (13%) and ascorbic acid (56%) caused decrease, while, $MgSO_4$ and $CaCl_2$ had no effect on immobilized enzyme. The enzyme electrode showed only 32% decrease during its use for 100 times over a period of 60 days at 4 °C.

1. Introduction

Uric acid is an end product of purine metabolism in human. A number of diseases and pathological disorders are related to the variations of uric acid concentration in body fluids (e.g. serum and urine), such as gout, arthritis [1], cardiovascular disease [2] and neurological diseases [3]. Consequently, uric acid determination is of paramount importance in the diagnosis of the diseases caused by disorder of purine biosynthesis and catabolism. Various techniques such as colorimetric [4], electrochemical [5], HPLC [6,7], chemiluminescence [8] and fluorescence methods [9] have been reported for the analysis of uric acid. However, these methods suffer from certain drawbacks such as time-consuming sample preparation and also oxidation of certain amounts of uric acid during the processing [4]. Since enzymes have an unique ability of molecular recognition, enzymatic colorimetric method improved the selectivity of uric acid determinations. So this method was chosen for routine clinical analysis [10,11]. Biosensors that employ immobilized enzyme (mostly oxidases), have been used for simple, sensitive, specific and rapid analysis of metabolites [12–17]. The immobilization of an enzyme is essential to stabilize its activity and get its reusability. Various immobilization methods such as adsorption, covalent bonding and entrapment have been employed to fabricate biosensors. Since 1970s, uric acid sensors have been developed employing uricase immobilized onto insoluble supports [18,19]. However, most researchers did not pay much attention to the methods of enzyme immobilization, as these could affect not only the sensitivity of the analysis, but also the shelf-life of the immobilized enzyme. Uricase has been immobilized on to various supports by different methods such as cellulose acetate membrane by adsorption [20], polyaniline film [7] and polypyrrole and polyaniline by physisorption [21] and chitosan membrane by crosslinking [22] polystyrene membrane by entrapment [23] and ZnO nano tetrapods [24]. Most of these immobilization methods were complicated and provided less stability, which limit the development and application of uric acid biosensors. We report herein a simple procedure of immobilization of uricase (purified from cowpea leaves) onto 'Araldite' membrane and its application in construction of a uric acid biosensor. 'Araldite' membrane, composed of epoxy resins having epoxy groups on both sides of single epoxy resin, forming a highly crosslinked network, is long-lived and water-permeable. The membrane has advantages such as high affinity for enzyme,

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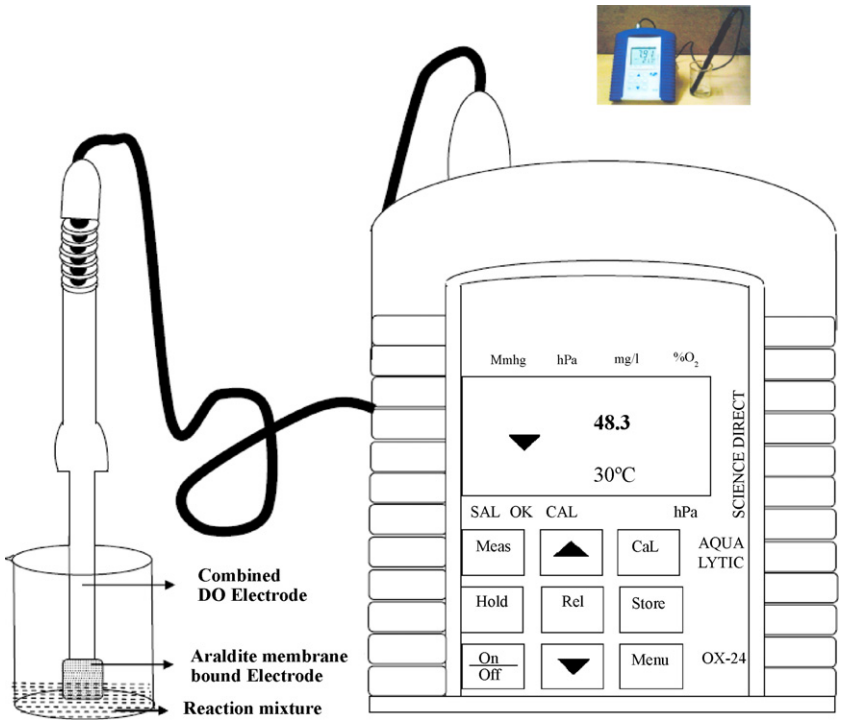


Fig. 1. A flow diagram of dissolved O_2 meter based urate biosensor employing Araldite membrane bound black-eyed cowpea uricase.

low cost, easy synthesis, high temperature stability, chemical resistance and porous in nature and thus an ideal material for making an enzyme electrode.

2. Materials and methods

2.1. Chemical and reagents

Sephadex G-100 and DEAE-Cellulose were from Sigma Chemical Co., St. Louis (USA). 'Araldite' as a source of epoxy resins and polyamine crosslinker, manufactured by Huntsman Advanced Materials Pvt. Ltd., Mumbai was purchased locally. Tris, uric acid, copper sulphate, and Folin-Clocalteu's reagent were from SISCO Research Laboratory Pvt. Ltd., Mumbai. All other chemicals used were of AR grade.

2.2. Collection of plant material

The seeds of cowpea (*Vigna unguiculata* var. V-240) were procured from NBPGER, IARI, New Delhi. The seeds of cowpea were soaked in deionized water overnight and sowed in the earthen pot containing loam soil. After 20 days, leaves were harvested and used immediately for the extraction of uricase.

2.3. Extraction and purification of uricase

Uricase from fresh cowpea leaves was extracted and purified by ammonium sulphate precipitation, ion exchange chromatography on DEAE cellulose and gel filtration on Sephadex G-100 as described [25]. The purified enzyme had an activity of 7.2 U/ml.

2.4. Assay of native uricase

Assay of uricase was carried out as described in ref. [26] and based on spectrophotometric measurement of allantoin generated from the oxidation of uric acid by uricase, which absorbs at 293 nm. The reaction mixture contained 5.8 μmol Tris–HCl buffer, pH 8.8 (saturated with O_2 gas bubbling for 5 min to avoid interference of atmospheric O_2). 0.36 μmol uric acid and 0.72 U of uricase in a total volume of 3.0 ml. The blank contained 2.9 ml of 20 mM Tris–HCl buffer pH 8.8 and 0.1 ml of uric acid (3.57 mM) and A_{293} was read in a UV and visible spectrophotometer (make: Shimadzu, Model 1700). Activity was calculated using following equation:

$$\text{Unit activity} = \frac{(\Delta\text{A at 293 in test solution} - \text{blank}) \times \text{test volume}}{12.6 \times \text{volume of enzyme taken}}$$

where 12.6 is extinction coefficient of uricase. ΔA is change in absorbance at 293 nm; test volume = 3.0 ml; volume of enzyme = 0.1 ml.

One enzyme unit is defined as amount of enzyme required to generate 1.0 μmol of allantoin from uric acid per min per ml at pH 8.8 at 25 °C.

2.5. Preparation of uricase–epoxy resin biocomposite membrane

The epoxy resin and hardener of 'Araldite' were mixed on a plastic (polythene) piece (size 4 cm × 4 cm) in 85:15 ratio at room temperature for 5 min. Two milliliter of purified enzyme was added to this mixture and spread equally to polymerize and crosslinked for 48 h. 'Araldite' membrane with entrapped uricase was stripped off the plastic piece and washed with 20 mM Tris–HCl buffer pH 8.8.

2.6. Scanning electron microscopy (SEM) of Araldite membrane

Araldite membrane with and without enzyme was subjected to scanning electron microscopy (SEM) at 'Sophisticated Analytical Instrumentation Facility' (DST), AIIMS, New Delhi to confirm the immobilization.

2.7. Construction and response measurement of dissolved O_2 metric urate biosensor

Uricase–epoxy resin biocomposite membrane was mounted over the sensing part of combined electrode of dissolved O_2 meter

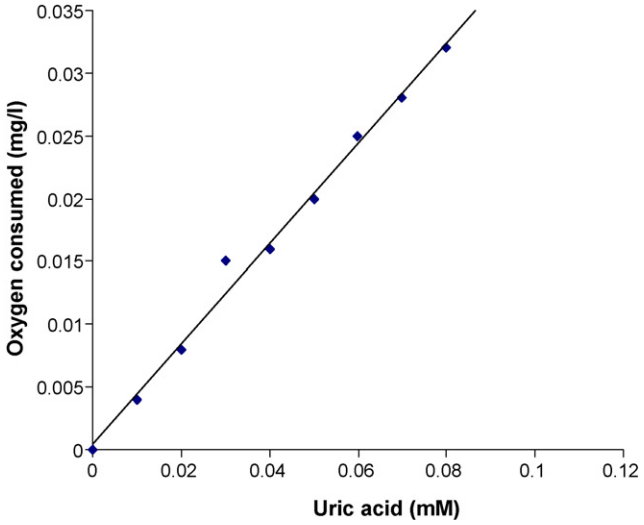


Fig. 2. Standard curve of uric acid concentration by biosensor using Araldite membrane bound black-eyed cowpea (*Vigna unguiculata*) uricase.

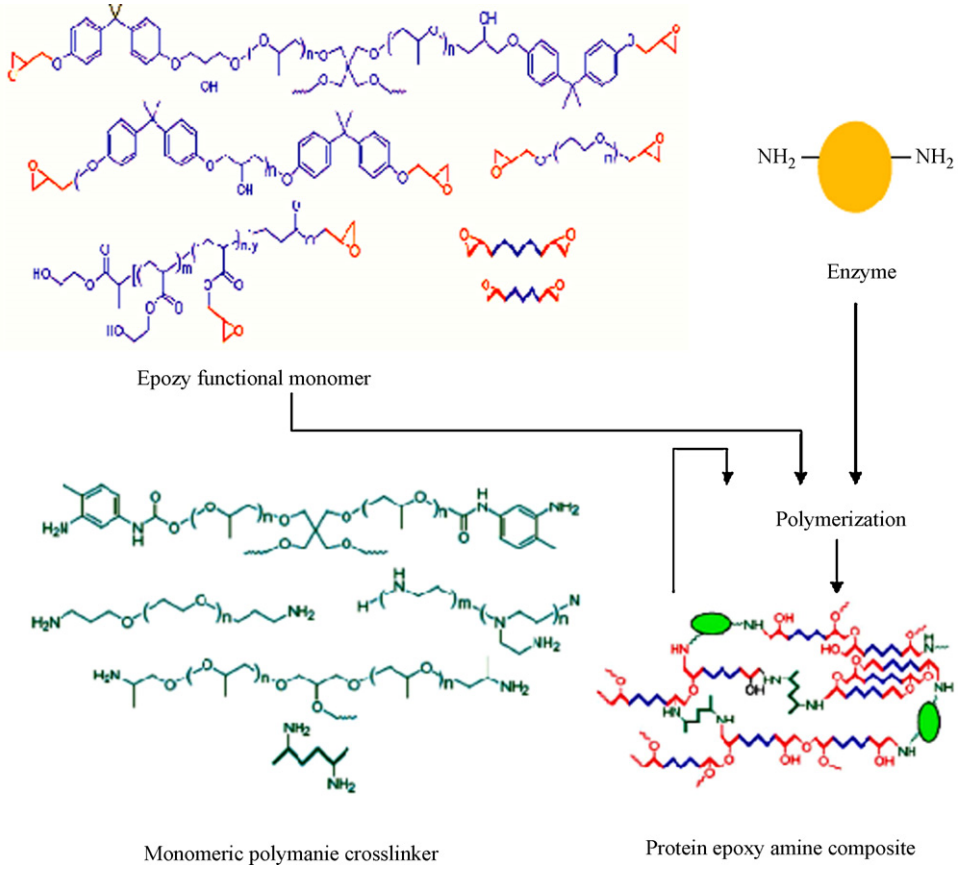
(make: Aqualytic Germany, Model: 0x53) with a parafilm. The electrode was connected to main apparatus of O_2 meter. To test this working enzyme electrode, it was immersed into 2.9 ml of 20 mM Tris–HCl buffer pH 8.8 (saturated with O_2 to avoid the intake of atmospheric O_2) in a 10-ml glass beaker. The meter was put on and the reading displayed on the screen was read in terms of mg/l and kept on hold. After adding 0.1 ml 1 mM uric acid solution, the dissolved O_2 content was measured again, till the reading became constant. The difference between two readings gave the amount of O_2 consumed in the enzyme assay (Fig. 1).

2.8. Optimization of urate biosensor

The optimum working conditions of biosensor/kinetic properties of uricase immobilized onto 'Araldite' membrane were studied and compared with those of free enzyme. To determine the optimum pH, the pH of reaction buffer was varied from 5.5 to 9.5 using 20 mM Tris–HCl buffer. Similarly, the optimum temperature was studied by incubating reaction mixture at different temperatures ranging from 15 to 40 °C at interval of 5 °C. The controls were also run at different temperatures. To study the effect of substrate concentration the uric acid concentration was varied from 0.05 to 1 mM. The K_m for urate and V_{max} were calculated from Lineweaver–Burk plot.

2.9. Determination of serum uric acid

Serum samples from apparently healthy persons and patients suffering from gout were collected from the local 'Pt.BDS PGIMS' hospital and stored at 4 °C until use. Uric acid content was determined in these serum samples by the present biosensor in the similar way as described under Section 2.7, for its response measurement, under its optimal working conditions except that uric acid solution was replaced by serum. Uric acid concentration in serum was extrapolated from the standard curve of uric acid between uric acid concentration (mM) and dissolved O_2 consumed (mg/l) prepared under optimal working conditions (Fig. 2).



Scheme 1. SEM of araldite Chemical reactions membrane involved in immobilization of enzyme onto epoxy resin membrane.

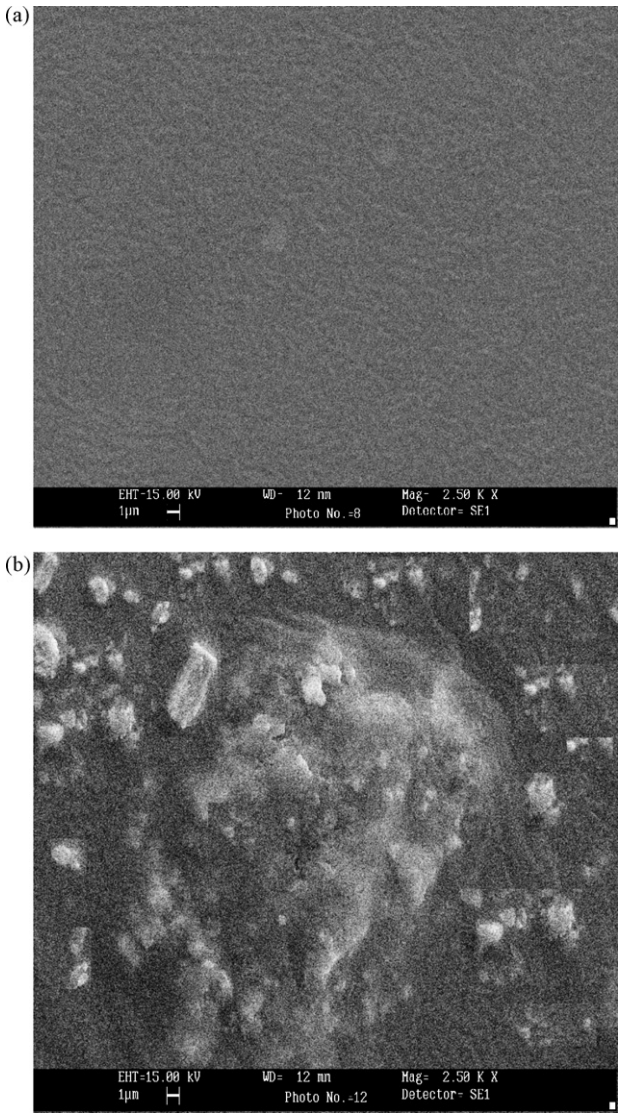


Fig. 3. (a) SEM of Araldite membrane without enzyme at 2500×. (b) SEM of Araldite membrane with enzyme at 2500×.

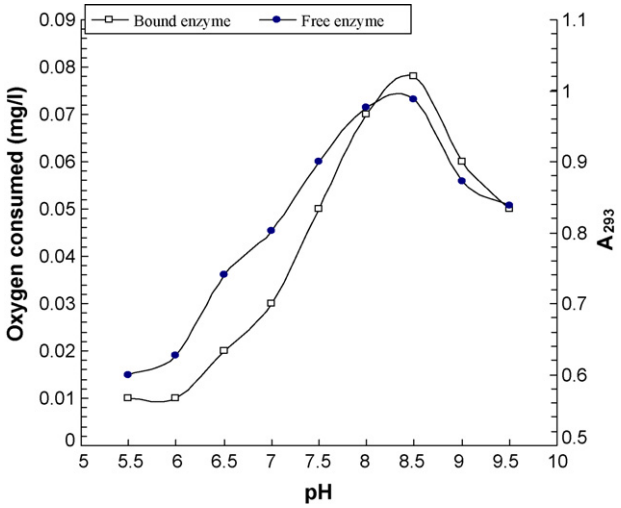


Fig. 4. Effect of pH on native/free and Araldite membrane bound black-eyed cowpea (*Vigna unguiculata*) leaf uricase.

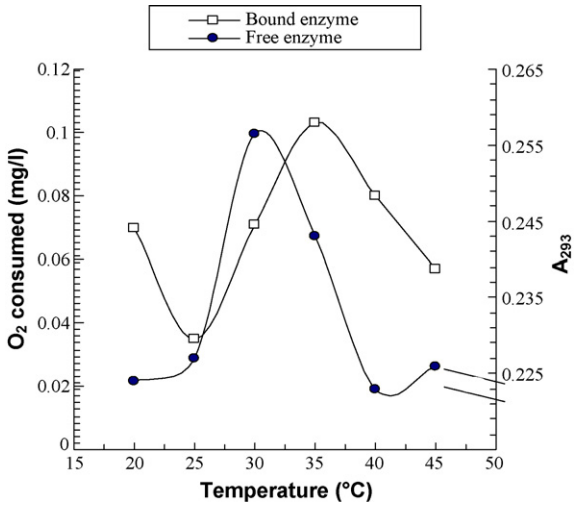


Fig. 5. Effect of incubation temperature on native/free and Araldite membrane bound black-eyed cowpea (*Vigna unguiculata*) leaf uricase.

2.10. Reuse of immobilized uricase

To reuse the enzyme electrode, it was washed 3–4 times with reaction buffer, dried in between folds of tissue paper. The enzyme electrode was stored in reaction buffer at 4 °C when not in use.

3. Results and discussion

3.1. Immobilization of cowpea leaf uricase onto 'Araldite' membrane

Uricase purified from leaves of 20 days old seedlings of black-eyed cowpea. Cowpea has been immobilized onto 'Araldite' membrane with 80% retention of initial activity of free enzyme with a conjugation yield of 0.056 mg/cm^2. The –OH groups of epoxy containing polymers react with bifunctional polyamine and –NH_2 groups of enzyme to form a network resulting into enzyme–epoxy amine resin composites. Scheme 1 represents the probable mechanism of immobilization of enzyme onto epoxy resin membrane which shows the covalent coupling of enzyme with the support [27,28].

3.2. Scanning electron microscopic (SEM) study of epoxy resin membrane

The surface of 'Araldite' membrane without and with enzyme was observed under a scanning electron microscope. High resolution scanning electron micrographs of Araldite membrane with immobilized enzyme had clusters along some beaded structures which were not observed in membrane without immobilized enzyme (Fig. 3a and b) which might be due to the both chemical coupling as well as adsorption of some enzyme. A change in surface morphology of membrane after immobilization process is an evidence of enzyme immobilization. The formation of clusters instead of regular globular beaded structures might be due to high concentration of uricase on the surface of Araldite membrane.

3.3. Construction of D.O. metric uric acid biosensor

A method is described for construction of a D.O. metric uric acid biosensor using 'Araldite' epoxy resin membrane bound uricase.

3.4. Kinetic optimization of uric acid biosensor

The optimum pH of immobilized uricase was pH 8.5, which is higher than that of its free form (pH 8.5) (Fig. 4). The optimum pH of immobilized enzyme was comparable to previous reports, e.g. optimum pH of polypyrrole film bound uricase (pH 8.8) [29], egg shell membrane bound uricase (pH 8.0) [30], copolymer of aniline and o-aminophenol bound uricase (pH 6.0) [31] and polypyrrole–ferrocene film bound uricase (pH 8.0) [32], and polyaniline bound *Aspergillus niger* uricase (pH 9.0) [20]. The optimum temperature of immobilized uricase was 35 °C, which is higher than that of free enzymes (30 °C) (Fig. 5). The optimum temperature in present epoxy resin bound uricase was same as that of polyaniline bound *Bacillus fastidious* uricase (35 °C) [33] but lower than that of alkylamine glass beads bound porcine liver uricase [34], pig liver uricase immobilized on aniline and o-aminophenol (45 °C; Pan et al. [31]), *Arthrobacter globiformis* uricase immobilized onto polypyrrole–ferrocenium film (55 °C; Çete et al. [32]) but higher than that of egg shell membrane bound uricase (30 °C) [30] and polypyrrole film bound uricase (25 °C) [25]. The effect of uric acid on the biosensor response was studied in the concentration range 0.05–1 mM. There was a hyperbolic relationship between biosensor response/immobilized uricase activity and uric acid concentration up to 0.6 mM (half of that of free form), after which it was constant. The decrease in K_m of uricase from 0.80 to 0.17 mM after immobilization revealed the increased affinity of the enzyme after immobilization. K_m for uric acid for uricase epoxy resin biocomposite membrane was lower than that of polypyrrole film bound uricase (K_m = 0.44 mM) [30], egg shell membrane bound uricase (K_m = 0.33 mM) [29] and for polypyrrole–ferrocenium film bound uricase (0.4 mM); however, V_{max} was increased from 0.02 to 0.08 mg/l after immobilization. The change in kinetics of uricase after immobilization might be due to many factors like conformational change due to chemical modification of the enzyme, steric effects, partitioning effects due to electrostatic or hydrophobic interactions between matrix and the low molecular weight species in solution causing modification of enzyme microenvironment and mass transfer or bulk diffusional effects arising from diffusional resistance to the transport of substrate from bulk solution to the catalytic sites as well as diffusion of reaction products back to the bulk solution [35]. A comparison of various kinetic properties of Araldite membrane bound uricase with those of uricase immobilized onto different supports is summarized in Table 1.

A simple, sensitive and rapid method for measurement of serum urate was developed using the present biosensor. The method has the advantage over other D.O. metric biosensors that it reads the sample within 10–12 s and is portable. The following analytical parameters were determined to evaluate the method.

3.5. Linearity

Immobilized uricase showed a linear relationship between dissolved O_2 consumed (mg/l) and uric acid concentration from 0.025 to 0.1 mM (Fig. 2).

3.6. Minimum detection limit

The present biosensor has the minimum detection limit of 4.25 μg/ml, which is lower than some earlier methods such as automated analysis using immobilized uricase (20 μg/ml) [36], enzymatic colorimetric method using free enzyme (25 μg/ml) [37], colorimetric method using commercially available porcine liver uricase (80 μg/ml) [4], colorimetric method using commercially available uricase (1.68 mg/dl) [38] but higher than few methods such as chemiluminiscence method using free enzyme (1 μg/ml) [39], voltametric and colorimetric titration method (2.0×10^{-5} M)

Table 1
A comparison of kinetic parameters of Uricase after immobilization onto Araldite membrane with those reported on other supports.

Properties	Bhargava et al. [4]	Pundir and Bhargava [34]	Sushma et al. [38]	Pan et al. [31]	Çete et al. [29]	Zhang et al. [30]	Present
Source of uricase	Porcine liver uricase	Porcine liver uricase	Recombinant bacterial uricase	Pig liver	*Arthrobacter globiformis*	*Arthrobacter globiformis*	Black-eyed cowpea leaf
Support for immobilization	Alkylamine glass beads	Arylamine glass beads	Alkylamine glass beads affixed on a plastic strip	Poly-o-aminophenol-aniline	Polypyrrole film	Egg shell membrane	Araldite membrane
Method of immobilization	Glutaraldehyde crosslinking	Glutaraldehyde crosslinking	Glutaraldehyde crosslinking	Entrapment	Glutaraldehyde crosslinking	Glutaraldehyde crosslinking	Hydrogen bonding
Optimum pH	8.8	8.8	8.8	8.0	8.0	8.0	8.5
Optimum temperature	40 °C	40 °C	40 °C	45 °C	25 °C		35 °C
V_{max}	Not reported	Not reported	0.068 μmoles H_2O_2/min/ml	Not reported	7.1×10^{-2} mM/min	Not reported	0.08 mg/l
K_m	Not reported	Not reported	0.15 mM	10.08 mmol dm^{-3}	0.44 mM	0.33 mM	0.17 mM
Working range	0.05–0.25 mM	10–80 μg/2.0 ml	0–48 μg/2 ml	0.001–0.5 mmol dm^{-3}	$(0.1–5.0) \times 10^{-5}$ M	4.0–6.40 μM	0.05–1 mM
Minimum detection limit	80 μg/ml	5.0 mg/0.1 ml	11.0 μg/0.1 ml	Not reported	5.0×10^{-7} M	2 μM	4.25 μg/ml
Mode of measurement	Colorimetric H_2O_2 estimation	Colorimetric H_2O_2 estimation	Colorimetric H_2O_2 estimation	Dissolved O_2 estimation	Amperometric	Dissolved O_2 estimation	Dissolved O_2 estimation

Table 2
Within and between assay coefficients of variation for determination of serum uric acid in the serum as measured by dissolved O_2 metric biosensor based on Araldite membrane bound black-eyed cowpea leaf uricase.

n	Uric acid (mg/l)	CV (%)
Within assay (6)		
4.28	4.47 ± 0.29	6.5
4.58		
4.58		
4.83		
3.99		
4.58		
Between assay (6)		
3.41		
3.10		
3.41	3.2 ± 0.16	5.0
2.41		
3.10		
3.41		

[36], oxygen metric method using commercial Arthrobactor uricase (2.0 μM) [30].

3.7. Recovery

The analytic recovery of added uric acid in serum at 8.9 and 9.8 mg/dl was 93.6 ± 2.34 and 87.18 ± 3.17%, respectively, which is comparable to the earlier reports [4,34,41].

3.8. Precision

To study the reproducibility and reliability of the present method, the uric acid content in same serum sample was determined six times a day (within batch) and after their one-week storage at −20 °C (between batch). The within and between batch coefficient of variation (CVs) were <6.5 and <5.0%, respectively (Table 2).

3.9. Accuracy

To test the accuracy of method, uric acid values in serum obtained by the present method (*y*) were compared with those obtained by standard enzymic colorimertric method using free enzyme. The serum uric acid values obtained by both the methods

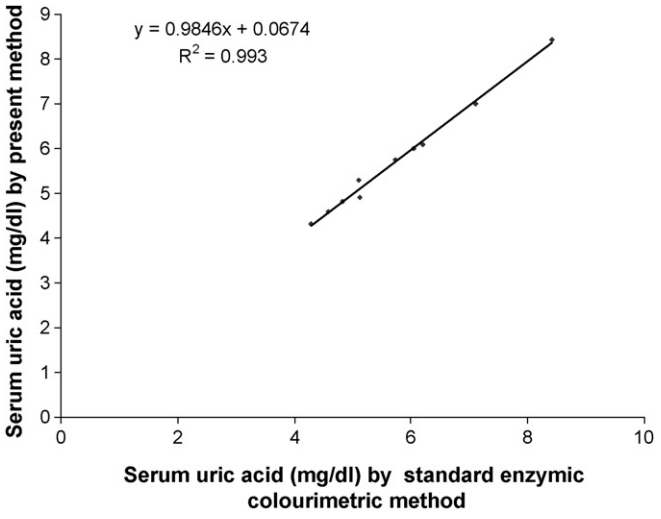


Fig. 6. Correlation between serum uric acid measured by standard enzymic colorimetric method (*x*) and present method (*y*) employing dissolved oxygen metric biosensor based on Araldite membrane bound black-eyed cowpea leaf uricase.

Table 3
Comparison of stability of uric acid biosensor with the present dissolved O_2 meter based biosensor using uricase epoxy resin biocomposite membrane.

Support for immobilization	Cellulose acetate (Gilmartin et al.) [18]	Chitosan membrane (Yao et al.) [39]	Polyelectrolyte multi-layer films (Hoshi et al.) [13]	ZnO nanorods (Zang et al.) [40]	Polyaniline (Kan et al.) [29]	Alkyl affixed on plastic strip (Sushma et al.) [32]	Co-polymer of o-amino phenol aniline (Pan et al.) [27]	Eggshell membrane (Zhang et al.) [26]	Araldite membrane (present work)
Time of study	7 days	7 days	30 days	20 days	60 days	21 days	50 days	90 days	60 days
% of initial response	86	85	60	80.5	82	50	81	89	88

matched with each other and showed a good correlation ($r = 0.98$), giving the following regression equation: $y = 0.984x + 0.0674$ (Fig. 6).

3.10. Interference

The aqueous solution 0.1 ml each of following metabolites and salts were added in the reaction mixture each: glucose (5 mM), cholesterol (3.87 mM), urea (2 mM), ascorbic acid (2 mM), NaCl (24 mM), $MgSO_4$ (8 mM) and $CaCl_2$ (4 mM). Among these, NaCl (2%), glucose (11%), cholesterol (13%) and urea (38%) caused decrease in activity; ascorbic acid caused maximum (56%) decrease in activity while $MgSO_4$ and $CaCl_2$ had no effect on activity [4] observed lesser, i.e. 2% decrease in activity by NaCl (2%) and glucose (2%) but almost similar inhibition (60%) by ascorbic acid in the activity of commercial uricase immobilized on alkylamine glass beads.

3.11. Serum urate determination

The uric acid content in serum as measured by the present biosensor was in the range 3.4–6.1 mg/dl with a mean of 4.92 mg/dl in apparently healthy males ($n = 6$), 2.4–5.14 mg/dl with a mean of 3.11 mg/dl in apparently healthy females ($n = 6$). This is comparable with normal accepted range of 3–7 mg/dl. The range of serum uric acid in healthy persons in present report is comparable with some earlier reports, i.e. the serum uric acid in apparently healthy adult male was found in the range of 46 μg/ml with a mean of 21 ± 0.013 by radioisotopic method using soluble uricase [37], 2–8 mg/dl [42] and recombinant bacterial uricase immobilized to plastic strip affixed alkylamine glass beads (2.5–5 mg/dl with mean of 3.7 mg/dl) [32]. The gout patient serum uric acid ranged from 9.4 to 13.6 mg/dl with mean of 10.4 mg/dl. This range is comparable to that of commercial uricase immobilized onto alkylamine glass beads (5.5–20 mg/dl) [4].

3.12. Reusability and storage

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 (Table 3).

4. Conclusion

The study has demonstrated that biosensor prepared by immobilization of uricase onto epoxy resin membrane provides a possibility to measure low concentration of uric acid with good sensitivity.

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