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Construction of dissolved O_2 metric oxalate biosensor using PVC membrane bound sorghum leaf oxalate oxidase

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A portable dissolved oxygen (DO) meter based oxalate biosensor has been constructed by mounting a polyvinyl chloride (PVC) membrane bound sorghum leaf oxalate oxidase on sensing part of combined electrode of an aqualytic DO meter. Biosensor showed optimum activity within 10-12 s at pH 5.0 and 30°C. A linear relationship was observed between oxalate conc. and O_2 consumption [1.0-10 µM (low conc. range), 0.1-1.0 mM (high conc. range)]. Biosensor gave mean value of oxalic acid in urine [22.5 mg/l in males (n=6) and 19.3 mg/l in females (n=6)], and in serum [3.2µM/l in males (n=6) and 2.9 µM/l in females (n=6)]. Biosensor is unaffected by physiological concentrations of Cl^- and NO_3^-.

Keywords: Dissolved oxygen meter, Oxalate biosensor, PVC membrane, Sorghum leaf oxalate oxidase

Introduction

Measurement of oxalate level in urine and blood is very important in diagnosis and medical management of primary and secondary hyperoxaluria, urinary stone disease, ethylene glycol poisoning, steatorrhoea and malabsorption[1]. Among various methods[2] available for oxalic acid determination, amperometric methods based on immobilized enzyme electrode are comparatively simpler, sensitive, specific and rapid[3-15]. Two types of amperometric oxalate biosensor employing immobilized oxalate oxidase (OOase) are reported: i) Dissolved oxygen (DO) meter based biosensor, which measures dissolved O_2 consumed in oxidation of oxalate by OOase[3-6]; and ii) Electrochemical biosensor, which monitors electrons, generated from H_2O_2, a product of OOase reaction[7-15]. OOase, purified from barley seedlings or beet stems and employed in all biosensors, is strongly inhibited by Cl^- and NO_3^- ions, normally found in biological fluids[16-19]. An OOase has been purified from leaves of 10-d old seedlings of grain sorghum and mature leaves of *Amaranthus spinosus*, which are insensitive to Cl^- and NO_3^-[20-21], and could be used in construction of enzyme electrode.

This study presents preparation of a portable aqualytic DO meter based oxalate biosensor employing a polyvinyl chloride (PVC) membrane bound sorghum leaf OOase and its application in urinary and serum oxalate determination.

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Experimental

Chemicals

4-Aminophenazone, glutaraldehyde (25%), oxalic acid, Sephadex G-200, DEAE-Sephacel, PVC, polyvinylpyrrolidone (PVPP) and peroxidase from horseradish were from Sigma Chemicals Co. USA. Isopropylmeristate and tetrahydrofuran were from Fluka, Folin-Ciocalteau's (FC). Phenol reagent, succinic acid, ammonium sulphate (enzyme grade), potassium chloride were from Sisco Research Laboratory Pvt. Ltd., Mumbai. All other chemicals were of AR grade.

Plant material and Extraction of Oxalate Oxidase (OOase)

Seeds of grain sorghum (*Sorghum vulgare* L variety Amarnath) were gift from M/s Nath seeds Pvt Ltd, Aurangabad, India. Seedlings (10 d old) of grain sorghum were raised[22] in laboratory and their leaves were separated with a scissor and stored immediately at -20°C until use. OOase from leaves was extracted[23]. Frozen leaves (350 g) were homogenized with chilled 0.01 M sodium phosphate buffer (pH 7.0) containing 1% PVPP in a chilled pestle and mortar in 1:3 ratio (w/v). Homogenate was filtered through double layer of muslin cloth and filtrate

was centrifuged at 15000 g at 4°C. Pellet was discarded and supernatant was collected and stored at 4°C until use.

Assay of Oxalate Oxidase (OOase)

Assay of OOase was carried out in a 15 ml test tube wrapped with black paper[23]. Reaction mixture containing 0.05 M sodium succinate buffer (1.7 ml, pH 5.0), 10^{-2}M CuSO4 (0.1 ml) and 15000g supernatant (crude enzyme, 0.1 ml) was pre incubated at 37°C for 5 min. Reaction was started by adding 10^{-2}M oxalate solution (0.1 ml). After incubation at 37°C for 10 min under continuous stirring, color reagent (1 ml) was added and kept at room temp for 15 min, in dark to develop color. Control was run in the same manner except that enzyme was heat denatured. In blank, reaction buffer replaced enzyme. A_{520} was read against blank in a Spectronic-20 (Milton &Roy, USA). H_2O_2 generated in reaction was extrapolated from standard curve between H_2O_2 concentrations vs A_{520}.

Color reagent consisted[24] of 4-aminophenazone (50 mg), solid phenol (100 mg) and horseradish peroxidase (1 mg) per 100 ml 0.4 M sodium phosphate buffer (pH 7.0). It was stored in amber colored bottle at 4°C and prepared fresh every week. One unit of enzyme is defined as amount of enzyme required to catalyze formation of 1.0 nmol of H_2O_2 per min per ml under standard assay conditions. Soluble protein in various enzyme preparations was determined by Lowry method[25].

Purification of Oxalate Oxidase (OOase)

A combination of 0-80% ammonium sulphate fractionation, gel filtration on Sephadex G-200 column (2.5 × 40 cm) in 0.02 sodium phosphate buffer (pH 7.2) and ion exchange chromatography on DEAE - Sephacel column (2.5 × 30 cm) using a linear gradient of 0.1M to 0.6M KCl in 0.02 M potassium phosphapte buffer (pH 6.7) resulted into 30-fold purification with 36% yield of crude OOase[23] (Table 1). Purified enzyme showed a major and a minor protein band in PAGE using AgNO3 as protein stain and a specific activity of 295 U/mg. Insensitivity of purified enzyme towards Cl^- (conc. range, 100-250 mM) and NO_3^- (conc. range, 1-8 mM) was confirmed[26].

Immobilization of Oxalate Oxidase on PVC Membrane

An artificial PVC membrane was prepared[8] by dissolving PVC (0.06 g) and isopropylmeristate (150 µl) in tetrahydrofuran (THF, 5 ml). Polymer solution was poured into a petridish (9 cm/ D) and rotated on a horizontal flat surface to get an even distribution of both polymer solutions over glass. Dish was covered with glass lid to allow slow and controlled evaporation of solvent and creation of a membrane of even thickness (0.2 mm).

Purified OOase was immobilized onto PVC membrane after BSA glutaraldehyde crosslinking[8]. To purified OOase (1 ml) containing 0.35U/1.2 mg protein, 300 mg BSA and 100 µl 2.5% glutaraldehyde in distilled water were added. Solution was poured over PVC membrane (2 cm × 2 cm). Another PVC membrane (2 cm × 2 cm) was placed on enzyme membrane and pressed mildly between two glass slides for 5 min. Resulting membrane laminate was washed with reaction buffer (0.05 M sodium phosphate, pH 7.0) and tested for OOase activity.

Construction, Testing and Standardization of Working Condition of Oxalate Biosensor

PVC membrane having immobilized OOase was mounted over sensing part of combined electrode of a DO meter (Aqualytic, Germany, Model: OX 53) with a parafilm M (American National Can™ Greenwich, USA). Enzyme electrode was connected to main apparatus of DO meter (Fig. 1). It was immersed into a mixture of 3.5 ml of 0.05 M sodium succinate buffer pH-5.0 (saturated with O_2 bubbling) and 10^{-2} M $CuSO_4$ (0.1 ml) solution in a 10 ml glass beaker to test activity. DO meter was put on and reading was kept on hold and then 10^{-2} M oxalic acid solution (0.2 ml) was added into mixture, shaken gently and dissolved O_2 was measured again, when reading became constant. Difference between two readings provided amount of dissolved O_2 consumed in enzyme reaction. For optimum pH of enzyme electrode, pH of reaction buffer was varied (4.0-6.0) at interval of pH 0.5 using 0.05 M sodium succinate buffer. For optimum temperature, reaction temperature was varied (20-40°C) at an interval of 5°C. To determine effect of substrate concentration, final concentration of oxalic acid in reaction mixture was varied (1.0-10 µM, low conc.; 0.1-1.0 mM, high conc.).

Determination of Urinary and Serum Oxalate by Oxalate Biosensor

First morning urine and serum samples of healthy persons and urinary stone formers of both sex and different age were collected from local Pt. BDS PGIMS hospital and stored at 4°C until use. Urine pH was adjusted to 5.0 with conc. HCl. Oxalate was analyzed in urine and serum as described for testing of enzyme electrode under its optimal assay conditions, except that

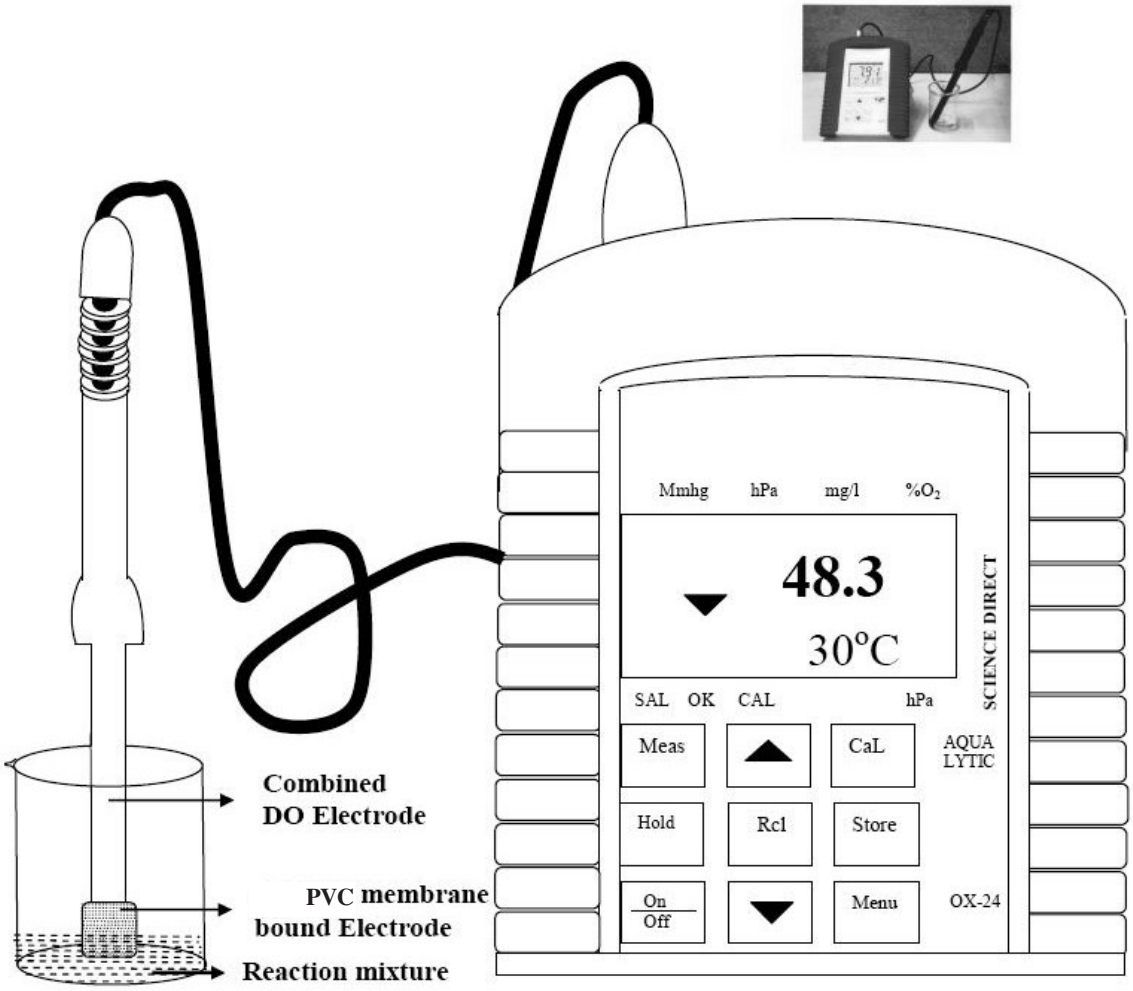

Fig 1—A flow diagram of dissolved O_2 meter based oxalate biosensor employing PVC membrane bound sorghum leaf oxalate oxidase [Inset: A photograph of DO metric oxalate biosensor]

Table 1-Purification of oxalate oxidase from grain sorghum leaves

Purification steps	Total vol ml	Protein mg/ml	Activity	Specific activity	Purification fold	Yield %
Crude enzyme	520	10.2	100	9.8	1	100
NH4(SO4)2 precipitation, 0-80%	45	7.2	510	70.8	7.2	44.1
Sephadex G-200	61	4.2	444	105.2	10.7	42.7
DEAE- Sephacel	53	1.2	354	295	30.1	36.1

oxalic acid solution was replaced by urine/serum. Oxalate (conc.) in urine/ serum was extrapolated from standard curve between oxalate concentrations versus O_2 consumed (mg/l) under optimal working conditions (Figs. 2 & 3).

Reusability and Storage of Electrode

To reuse electrode, it was washed with distilled water 3-4 times at room temperature followed by reaction buffer and then dried with a tissue paper. Enzyme electrode was stored in 0.05M sodium succinate buffer (pH 5.0) at 4°C when not in use.

Evaluation of Oxalate Biosensor

Solid oxalate was added into 6 urine samples (30 mg/l each) and oxalate content in urine sample (before and after addition of oxalate) was determined by present oxalate biosensor. Precision was studied by measuring oxalic acid in 6 urine samples in one run (within day) and after storage of one week at -20°C

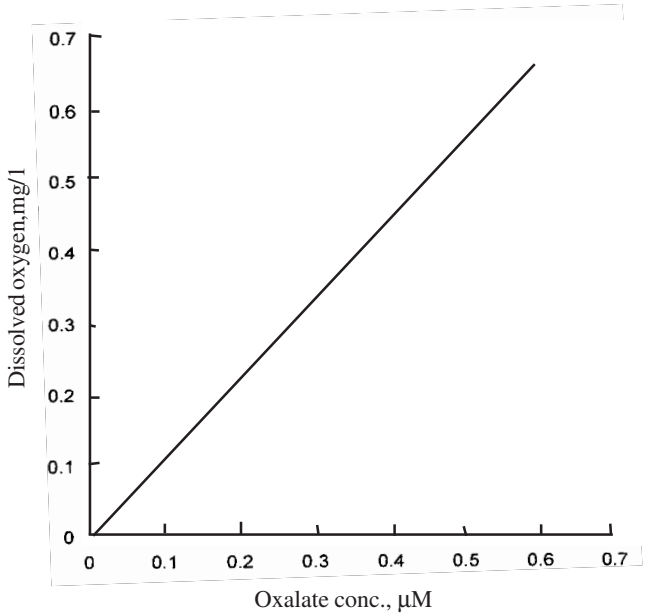

Fig 2—Standard curve between oxalate (lower conc. range) and response of DO metric oxalate biosensor using PVC membrane bound sorghum leaf oxalate oxidase

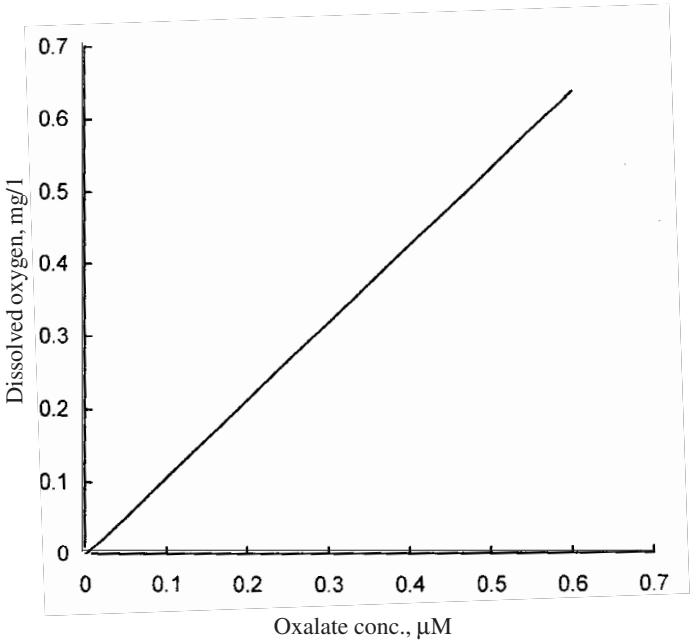

Fig. 3—Standard curve between oxalate (higher conc. range) and response of DO metric oxalate biosensor using PVC membrane bound sorghum leaf oxalate oxidase

(between day variation) and within and between batch coefficient of variation (CV) were calculated. To study accuracy of oxalate biosensor, oxalate value in 20 urine samples of healthy and stone formers were determined by present method and by standard enzymic colorimetric method (Sigma) and correlated using regression equation.

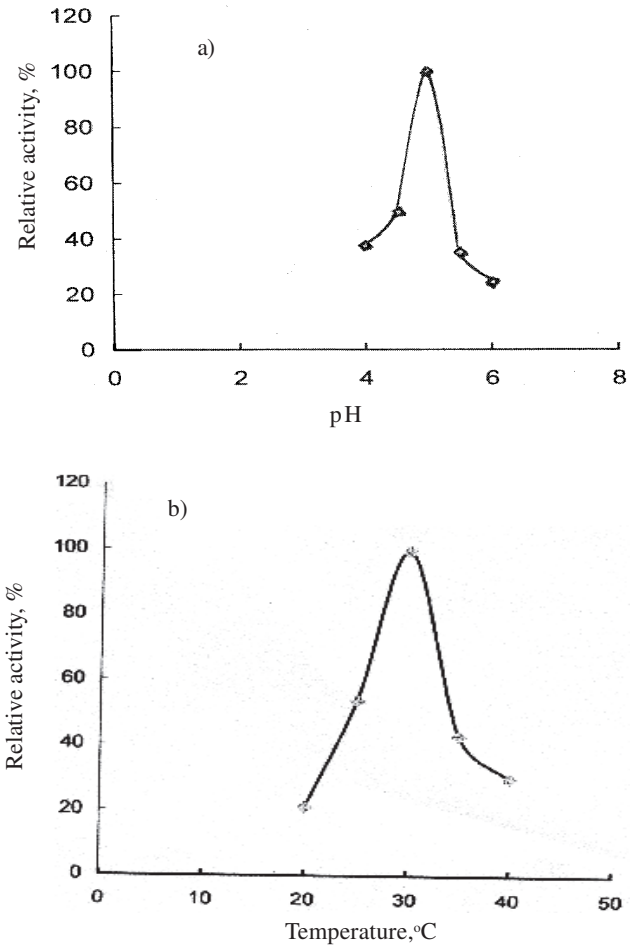

Fig. 4—Effect on activity of PVC membrane bound sorghum leaf oxalate oxidase of: a) pH; b) Incubation temperature

Results and Discussion

Standardization of Working Conditions Of Oxalate Biosensor

Optimum pH

Biosensor / immobilized enzyme showed maximum activity at pH 5.0 (Fig. 4a), which is slightly lower than that of free enzyme (pH 5.5)[28]. A slight decrease in optimum pH of barley OOase from pH 3.2 to 2.8 has also been reported after immobilization on PVC membrane[9]. Optimum pH (3.2 -3.5) of DO metric biosensor employing barley OOase immobilized onto gelatin has been reported[5].

Effect of Incubation Temperature

Biosensor/immobilized enzyme exhibited maximum activity at 30°C (Fig. 4b), which is 10°C less than that of free enzyme[28], might be due to decrease in activation energy (Ea) after being crosslinked with glutaraldehyde and adsorbed onto PVC membrane. A decrease in

Table 2—Urinary and serum oxalate determination in apparently healthy individuals (N) and stone formers (SF)by dissolved oxygen meter based oxalate biosensor using PVC membrane bound sorghum leaf oxalate oxidase

S No.	Sex	Urinary oxalate, mg/24 h		Serum oxalate, μM	
		N (age in y)	SF (age in y)	N (age in y)	SF (age in y)
1	M	15.3 (26)	33.3 (40)	2.5 (22)	5.0 (40)
2	M	24.3 (35)	41.4 (35)	3.3 (28)	5.1 (35)
3	M	23.4 (28)	42.3 (51)	2.5 (18)	5.2 (51)
4	M	27.0 (29)	-	4.2 (32)	-
5	M	23.5 (25)	-	3.5 (27)	-
6	M	21.5 (30)	-	2.8 (25)	-
7	F	13.5 (23)	35.1 (37)	2.2 (20)	5.3 (37)
8	F	24.3 (31)	37.8 (26)	3.0 (35)	6.0 (26)
9	F	20.7 (18)	36.0 (43)	3.6 (29)	5.8 (43)
10	F	18.9 (20)	-	2.9 (28)	-
11	F	19.0 (25)	-	-	-
12	F	19.6 (27)	-	-	-
Mean in males		22.5	39.0	3.2	5.1
Mean in females		19.3	36.3	2.9	5.7

optimum temperature had also been reported for barley seedling enzyme after immobilization on pig intestine membrane[4] and gelatin[5].

Response Time

Biosensor showed optimum response within (10-12 s), which is lower than earlier Clark type or YSI dissolved oxygen meter based biosensors employing barley enzyme (30s)[5] and spinach leaf enzyme (2-3 min in lower conc. range and 6-7 min in high conc. range)[6].

Effect of Substrate Concentration

As concentration of oxalate was increased, oxygen consumption also increased .A linear relationship between oxalate concentration (low 1-10 μM, high 0.1-1.0 mM) and consumption of dissolved O_2 was used as standard curve for determination of oxalate in serum and urine (Figs 2 & 3).

Application of Oxalate Biosensor

Present method, developed for simple, sensitive and rapid quantitative analysis of oxalate in urine and serum, has advantage over other DO metric biosensor as it reads sample within 10-12 s and does not require pretreatment of urine and serum samples for removal of Cl^- and NO_3^- .

Evaluation of Biosensor

Minimum Detection limit

Minimum detection limit of biosensor (1.0 μM), is lower than that for DO metric biosensors employing barley seedling enzyme (5.0 μM) and spinach leaf tissue (10 μM)[6].

Analytical Recovery and Precision

Analytical recovery of added oxalate (30 mg/l) in urine was 92%. Within and between batch CV of oxalate determination in urine were <10% and <8.5% respectively showing high reproducibility of the method. These values are comparable to those by spinach tissue homogenate[6].

Correlation with Sigma Kit Method

To test accuracy of the method, oxalate values in 20 urine samples of healthy and stone formers by present method (y) were compared with those obtained by enzymic colorimetric method (Sigma). Correlation coefficient (r^2) was 0.98 with regression equation being 0.984x-0.128 (Fig. 5) indicate high accuracy of the method.

Determination of Urinary and Serum Oxalate

Oxalate content in first morning urine samples of healthy adults [15.3-27.0 mg/l; av 22.5 mg/l in males (n=6); 13.5 -24.3 mg/l; av 19.3 mg/l in females (n=6)] and urinary stone formers (33.3 -41.4mg/l, av 37.6 mgl) was determined (Table 2) by present biosensor. Oxalate content in urinary stone formers was about two times higher than those in healthy adults and in agreement with those reported (41.2 mg/l) by alkylamine glass bound sorghum leaf oxalate oxidase[28]. Oxalate content in

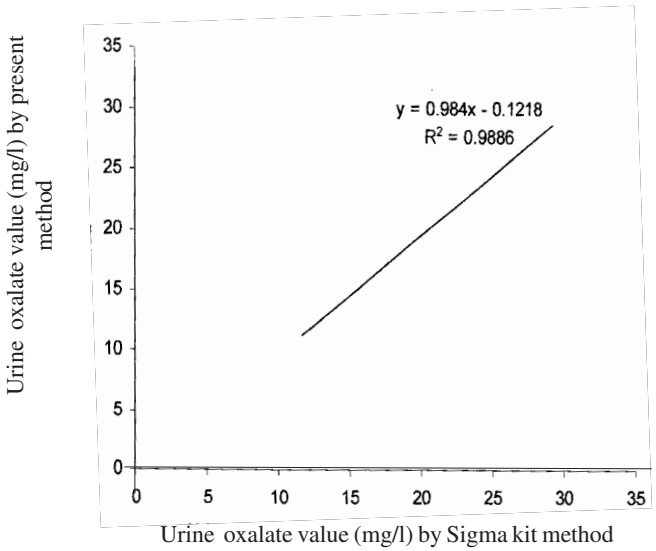

Fig. 5—Correlation between urine oxalate value as determined by Sigma kit method employing soluble enzymes (X-axis) and present biosensor method (Y-axis)

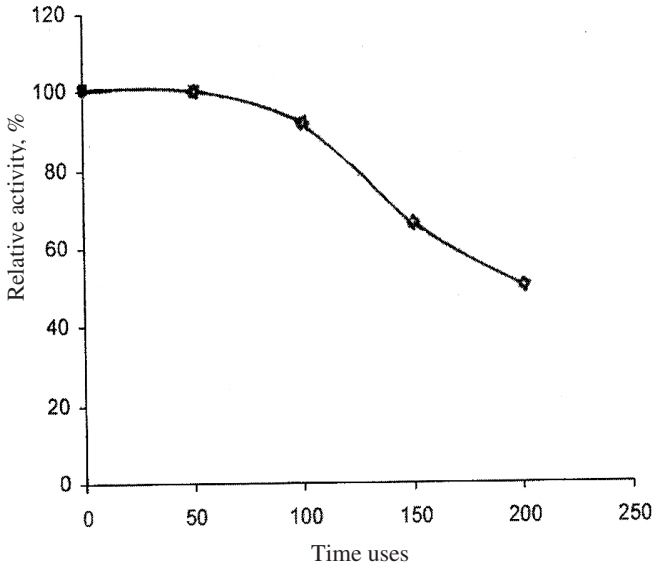

Fig. 6—Reusability of enzyme electrode

Table 3—A comparison of dissolved oxygen metric oxalate biosensor using membrane bound oxalate oxidase from different sources

Sources of oxalate oxidase	Barley[4] root (*Hordeum vulgare*)	Spinach[5] (*Spinacea oleracea*)	Grain sorghum leaves (Present)
Support for immobilization	Teflon membrane	Teflon membrane	PVC membrane
Method for immobilization of oxalate oxidase	Copolymerization with gelatin & glutaraldehyde coupling dissolved O_2	Glutaraldehyde crosslinking	Glutaraldehyde coupling
Type of DO meter used	YSI	YSI	Aqualytic
Linearity	5 x 10^{-6} to 2 x10^{-4} M	1 x 10^{-5} to 10 x 10^{-5} M	1 x 10^{-6} to 1 x 10^{-3} M
Response time	30 s	2-3 min (lower conc.) 6-7 min (higher conc.)	10-15 s
Storage life	90 days	2 month	75 days
No. of possible assays	400	-	200
Optimum pH	3.2	3.5	5.0
Optimum temperature	35°C	37°C	30°C

freshly collected serum samples of healthy individual [2.3 -4.2µM/l av 3.2 µM/l in males (n=6); 2.2 -3.0 µM/l, av of 2.9 µM/l in females (n=6)] and serum urinary oxalate in stone formers (5.0 -6.0 µM/l, av 5.4 µM/l). was measured by present biosensor (Table 2). A comparison of present biosensor with those employing barley OOase immobilized onto gelatin through glutaraldehyde crosslinking[5] and a spinach (*Spinacea oleracea*) tissue homogenate based biosensor[7] is summarized (Table 3).

Storage Stability and Reusability

Biosensor was used 200 times over 75 days, when stored at 4°C. However, it lost 50% of its initial activity after 200 uses (Fig. 6).

Conclusions

A portable DO metric oxalate biosensor was constructed employing a PVC membrane bound sorghum leaf OOase. Biosensor showed optimum response within 10-12 s at pH 5.0 at 30°C. It was employed for

measurement of oxalic acid in urine and serum. Biosensor was used 200 times over 75 days, when stored at 4°C and has advantages over previous biosensors that it is unaffected by physiological concentrations of Cl^- and NO_3^- and reads sample within 10-12 s.

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