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Fabrication of an amperometric ascorbate biosensor using egg shell membrane bound *Lagenaria siceraria* fruit ascorbate oxidase

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ABSTRACT

An ascorbate oxidase (EC 1.10.3.3) purified from *Lagenaria siceraria* fruit was immobilized onto egg shell membrane through glutaraldehyde coupling with 73.3% retention of its initial activity and a conjugation yield of 0.097 mg/cm^2. The membrane consisting of ascorbate oxidase was mounted over an Au electrode to construct a working electrode for ascorbate biosensor. The biosensor showed optimum response i.e. current in mA within 10 s at pH 6.0, 40 °C and 0.6 V using Ag/AgCl reference and Cu wire as auxiliary electrode. There was a linear relationship between L-ascorbic acid concentration in the range 1×10^{-5} M and 4×10^{-4} M and current. The biosensor was employed for determination of L- ascorbic acid in serum, fruit juices and vitamin C tablets. The analytical recovery of added ascorbate in sera was 98.2% and 96.7%. Within batch and between batch coefficients of variations (CV) in ascorbate of sera were <3.6% and <4.49% respectively. L-Ascorbate values obtained for fruit juices and vitamin C tablets by present method and by DCPIP (2,6-dichlorophenolindophenol) method, showed a good correlation (r = 0.993). The biosensor has advantages such as fast response time (10 s), good repeatability (200 assays) and long-term stability (50% of its initial sensitivity after 4 months of storage).

1. Introduction

Ascorbic acid or vitamin C being reductive in nature has widespread use as an antioxidant agent in foodstuffs and soft drinks. As an antioxidant, vitamin C's primary role is to neutralize free radicals. Since ascorbic acid is water soluble, it can work both inside and outside the cells to combat free radical damages. Vitamin C is an excellent source of electrons therefore it can donate electrons to free radicals such as hydroxyl and superoxide radicals and quench their reactivity" [1]. Vitamin C plays an important role in a number of metabolic functions including the activation of vitamin B, folic acid, the conversion of cholesterol to bile acids and the conversion of the amino acid, tryptophan to the neurotransmitter, serotonin. It is used as therapeutic agent in many diseases and disorders. Vitamin C protects the immune system by reducing the severity of allergic reactions and helps fight off infections [2]. Deficiency of vitamin C is known to cause anemia, scurvy, infections, bleeding gums, muscle degeneration, poor wound healing, atherosclerotic plaques and capillary hemorrhaging, neurotic disturbances consisting of hypochondriasis, hysteria and depression followed by decreased psychomotor performances [3,4]. Among the various methods available for determination of ascorbic acid such as colorimetric [5], spectrophotometric methods [6], high performance liquid chromatography [7] and sequential injection spectrophotometry [8], electrochemical methods are considered as one of the most potential approach, because of its high sensitivity, simplicity, rapidity, sufficiently short response time and durability. However, direct oxidation of ascorbic acid at bare electrodes is irreversible and requires a high overpotential. This high overpotential results in electrode fouling, poor reproducibility, low selectivity and low sensitivity. Thus, various chemically modified electrodes have been proposed for determination of ascorbic acid such as β-cyclodextrin–ferrocene inclusion complex modified carbon paste electrode [9], glassy carbon modified with nickel(II) macro cycle containing dianionic tetraazaannulene ligand [10], nylon net membrane [11], electrochemically etched platinum microelectrode [12], multilayer films of carbon nanotubes and redox polymer on screen-printed carbon electrodes [13], polypyrrole nanowire modified electrode [14], carbon nanotube-modified carbon fiber microelectrodes [15], micelle membrane coated on both aminated glassy carbon electrode and gold electrode [16], Cu(II) zeolite-modified electrode [17] and dopamine using a poly(acriflavine)-modified electrode [18]. In all these electrodes, ascorbate oxidase has been immobilized onto various supports either through absorption or entrapment or encapsulation, which allows leakage of enzyme resulting into low stability of electrode. Covalent immobilization of enzyme not only overcomes this problem but also leads to better bimolecular activity and greater stability. We have reported a method for covalent immobilization of enzymes onto egg shell membrane, which is expected to overcome this problem. Further

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an egg shell membrane has excellent gas and water permeability, biocompatibility, low cost and stability [19]. Being an inert support, it has been used as a good support for immobilization of enzyme in biosensor construction [20].

The present report describes the construction and application of an ascorbate biosensor by immobilizing an ascorbate oxidase purified from bottle gourd fruit onto egg shell membrane and then mounting this membrane onto Au electrode.

2. Materials and methods

2.1. Reagents

Sephadex G-100, DEAE-Sephacel and glutaraldehyde (grade 1, 25%) from Sigma–Aldrich, ammonia and nickel chloride from Sisco Research Laboratory (India) were used. Leghorn white eggs were purchased from local market. All other chemicals were of analytical reagent (AR) grade. The commercial vitamin C tablets (marketed under the brand name "Lamcea and Becozyme c forte" manufactured by Bayer, EU, Turkey) and fruits (lemon, grape, orange and apple) were purchased from local market. Fresh serum samples of healthy individuals were collected from hospital of Pt. BDS University of Health & Medical Science, Rohtak and stored at −20 °C until use. Fresh green fruits of bottle gourd (*Lagenaria siceraria*) of 10–15 cm diameter were collected from nearby village during the month of June–July (30 ± 5 °C) in ice bath, washed in distilled water and stored at 4 °C until use.

2.2. Extraction and purification of ascorbate oxidase

Ascorbate oxidase was extracted and purified from fresh green fruits of bottle gourd (*L. siceraria*) using a combination of 65% ammonium sulphate precipitation, gel filtration on Sephadex G-100 and ion-exchange chromatography on DEAE-Sephacel as described in [21] with slight modification. The purified enzyme exhibited a single band in simple polyacrylamide gel electrophoresis (PAGE) using coomassie blue as protein stain, indicating its apparent homogeneity (results not given). The purified enzyme had an activity 9.6 unit/ml.

2.3. Assay of ascorbate oxidase

The assay of ascorbate oxidase was carried out as described by Oberbacher and Vines [22] with slight modification. The reaction mixture contained 290 μmol phosphate/EDTA buffer (pH 5.6), 0.5 μmol L-ascorbic acid and 100 μg of enzyme in a total volume of 3.1 ml. The blank contained 3.00 μmol of phosphate/EDTA buffer pH 5.6 and 0.5 μmol ascorbic acid in a total volume of 3.1 ml. A_{265} was read in a UV and visible spectrophotometer (Make: Shimadzu 1700, Japan). The activity of enzyme was calculated as follow:

$$\text{Activity (U/ml)} = \frac{(\Delta A_{265}/\text{min}) \times 3.1 \times \text{dilution factor}}{e \times 0.1}$$

where e = 13.386 (extinction coefficient of dehydroascorbate); total volume = 3.1; enzyme volume = 0.1 ml.

2.4. Pretreatment and activation of egg shell membrane

It was carried out as described in [23]. An egg was covered with 150 ml of 3 M HCl in a 1 l flask. As the acid dissolves the egg, carbon dioxide gas is liberated and white foam of calcium chloride rises slowly in the flask. When the reaction was completed egg shell membrane was carefully separated from egg after removing egg yolk. Egg shell membrane was washed with distilled water to remove all residual particles and then cut into rectangular pieces (dimension 4 cm × 2 cm). A piece of membrane was washed again with distilled water and transferred to cleaned test tube containing reagent A (10 ml of liquid ammonia and 50 mg of nickel chloride). After incubation in reagent A for 5 h, membrane was washed with distilled water to remove excess of reagent A. This pretreated membrane was mounted onto one end of Au electrode (1.5 cm × 0.05 cm) with a parafilm. Affixed egg shell membrane was treated with reagent B (2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.0) for 2 h at ambient temperature (RT). The electrode with affixed membrane was taken off from reagent B and washed many times with distilled water.

2.5. Immobilization of enzyme/preparation of working electrode

Ascorbate oxidase was immobilized on pretreated egg shell membrane through glutaraldehyde coupling as described in [23]. The enzyme (25 μl) was placed onto activated egg shell membrane and kept at 4 °C in dark for 10 h to allow covalent coupling between enzyme and membrane. The membrane was washed 4–5 times with buffer (0.1 M phosphate EDTA buffer, pH 6.0) to remove unbound enzyme. The immobilization occurred by coupling of $-NH_2$ groups on surface of enzyme with -CHO groups introduced on egg membrane through glutaraldehyde. Fig. 1 depicts the chemical reaction involved in immobilization of enzyme(s) on egg shell membrane.

2.6. Construction of amperometric ascorbate biosensor and response measurement

An amperometric ascorbate biosensor was constructed by connecting the working electrode with a silver/silver chloride (Ag/AgCl) reference electrode and Cu as auxiliary electrode through a three terminal electrometer (Keithley, 6215A/E Japan). To test the activity of the three-electrode system, it was immersed into a mixture of 2.9 ml 0.1 M phosphate–EDTA buffer, pH 5.6 and 0.1 ml ascorbic acid (0.005 M). The electrode was polarized applying different potential in the range 0.1–0.8 V and the current (mA) generated, was measured. The following electrochemical reactions occur during measurement:

$$L\text{-Ascorbic acid} + \frac{1}{2}O_2 \xrightarrow{\text{ASOD}} \text{dehydroascorbic acid} + H_2O$$

$$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$$

where ASOD = ascorbate oxidase.

2.7. Optimization of ascorbate biosensor

The optimum working conditions of biosensor/kinetic properties of immobilized ascorbate oxidase were studied at 0.6 V (voltage at which maximum current was generated) and compared with those of free enzyme. To determine the optimum pH, the pH of reaction buffer was varied from pH 3.0 to 6.5 using the following buffer; each at a final concentration of 0.1 M: pH 3.0–5.0 sodium citrate and pH 5.5–6.5 phosphate–EDTA buffers. Similarly, the optimum temperature was studied by incubating reaction mixture at different temperatures ranging from 15 °C to 50 °C at an interval of 5 °C in a controlled temperature water bath. To study response time, the current was measured at 2 s, 4 s, 6 s, 8 s, 10 s and 12 s. To study the effect of substrate concentration, the ascorbic acid concentration was varied from 1 μM to 500 μM. K_m and I_{max} were calculated from Lineweaver–Burk (LB) plot.

2.8. Amperometric determination of ascorbic acid in serum and fruit

Fresh serum samples (0.5 ml) from apparently healthy persons were collected at local Pt BDS Postgraduate Institute of Medical Science hospital, Rohtak. To 0.2 ml of serum sample, 0.07 mg $NaNO_2$ and then 1 ml phosphate–EDTA buffer were added. To prepare

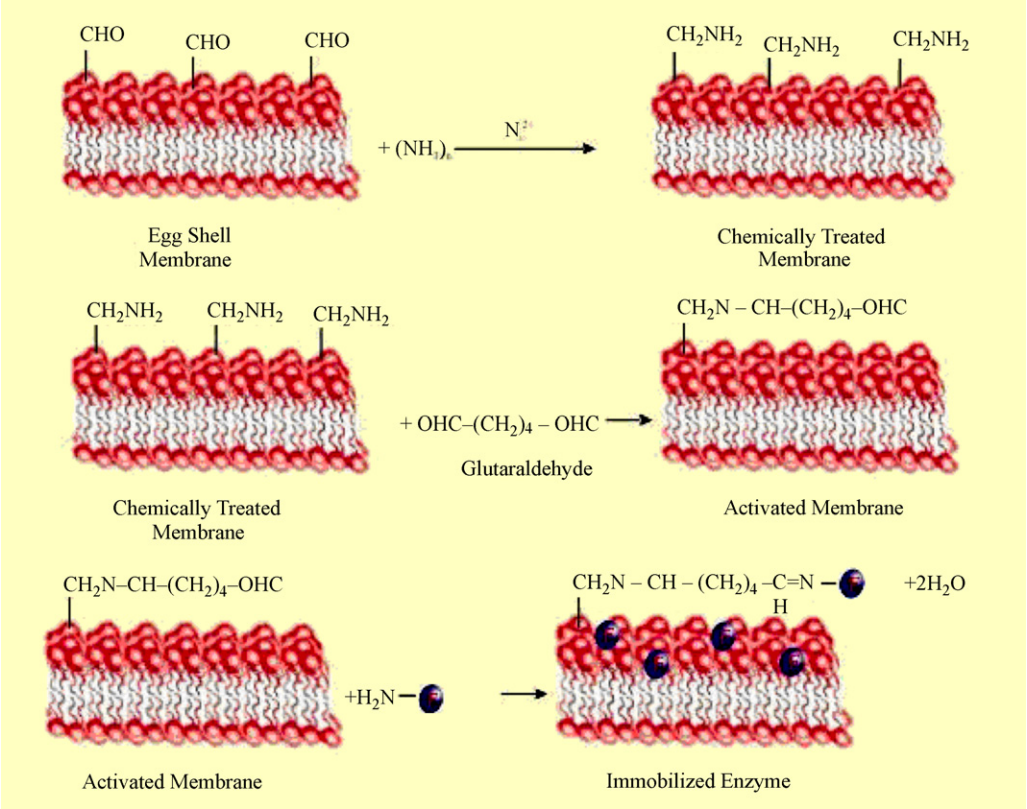

Fig. 1. Schematic diagram of immobilization of enzyme (ascorbate oxidase) onto egg shell membrane.

fruit sample, the average sized fruits (lemon, orange, grapefruit and apple) were peeled off and their juices were collected separately by squeezing them. The juice was centrifuged at 8000 × *g* for 5 min and supernatant was collected. The biological fluids were analyzed as described for response measurement of biosensor except that ascorbic acid solution was replaced by biological sample. The ascorbate content in biological fluid was calculated from standard curve between ascorbate concentration vs. current in mA (Fig. 2).

2.9. Amperometric measurement of ascorbate in Vit-C tablet

To measure L-ascorbic acid in vitamin C tablets, Lamcea (1000 mg L-ascorbic acid tablet) and Becozyme c forte (500 mg L-ascorbic acid tablet) was dissolved in the phosphate/EDTA buffer (0.1 M, pH 6.0) separately after grinding it in a pestle mortar. The contents of L-ascorbic acid in both vitamin C tablet solutions was determined by the present biosensor as described for its testing under optimal working conditions except that ascorbate was replaced by dissolved tablet.

2.10. Effect of serum and fruit substances

To study the effect of important serum metabolites in the present method, 0.1 ml aqueous solution of following compounds was added to the reaction mixture each, at a final concentration of 1×10^{-4} M: glucose, fructose, cysteine, sucrose, urea, cholesterol, starch, sucrose, sodium chloride, tartaric acid and lactose.

2.11. Reuse of enzyme electrode

To reuse the enzyme electrode, it was washed 3–4 times with the reaction buffer (0.1 M phosphate/EDTA buffer pH 6.0), dried in between folds of tissue paper before its use in next assay. The enzyme electrode was stored in the same buffer at 4 °C, when not in use.

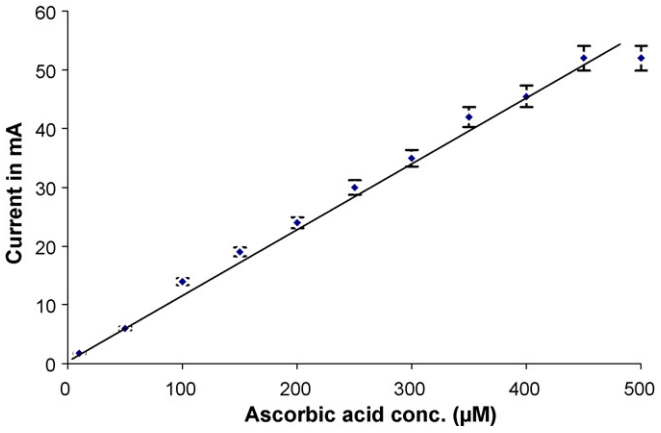

Fig. 2. Standard curve of current (mA) vs. ascorbate (μM) by ascorbate oxidase biosensor using egg shell membrane bound bottle gourd fruit ascorbate oxidase.

3. Results and discussion

3.1. Immobilization of L. siceraria fruit ascorbate oxidase onto egg shell membrane

An ascorbate oxidase purified from fresh green fruits of bottle gourd (*L. siceraria*) was immobilized covalently onto egg shell membrane with 73.3% retention of initial activity of free enzyme with a conjugation yield of 0.097 mg/cm^2. Immobilization of enzyme onto egg shell membrane was confirmed by scanning electron microscopy (results not shown).

Table 1
A comparison of some properties of ascorbate biosensors based on plant ascorbate oxidase.

Property	Marques et al. [25]	Fatibello-Filho and Vieira [26]	Akyilmaz and Dinçkaya [28]	Wang et al. [16]	Present
Source of ascorbate oxidase	*Cucumis sativus*	*Cucurbita pepo medullosa*	*Cucumis sativus.*	*Cucumis sativus*	*Lagenaria siceraria*
Support for immobilization	Alkylamine glass beads	Carbon paste electrode	Teflon membrane	Micelle membrane	Egg shell membrane
Method of immobilization	Covalent coupling	Adsorption	Absorption	Absorption	Covalent coupling and adsorption
Optimum pH	5.5	6.5	7.5	5.5	6.0
Optimum temperature (°C)	25	25	35	25	40
Mode of measurement	Dissolved O_2	Current	Current	Current	Current
Response time	2–3 min	20 s	45 s	30 s	10 s
Minimum detection limit	ND	2.2×10^{-5} mol l^{-1}	5.0×10^{-5} and 1.2×10^{-3} mol l^{-1}	ND	1×10^{-5} mol l^{-1}
Range (mol l^{-1})	6.25×10^{-7} to 5.0×10^{-8}	2.0×10^{-4} to 5.5×10^{-3}	4.0×10^{-4} to 1.0×10^{-3}	5×10^{-6} to 4×10^{-4}	1×10^{-5} to 4×10^{-4}
Storage life at 4 °C (days)	60	60	60	30	120

ND = not detected.

3.2. Optimization of biosensor

To optimize biosensor, the kinetic properties of immobilized enzymes were observed under different physiological conditions like change in pH of reaction mixture, temperature and time of incubation etc. The optimal pH of biosensor/immobilized ascorbate oxidase was 6.0, which is slightly higher than that of free enzyme (pH 5.5) [24]. The optimum temp of biosensor/immobilized ascorbate oxidase was 40 °C, which is similar to that of free enzyme (40 °C) [24]. The rate of reaction of immobilized ascorbate oxidase was linear up to 10 s, after which it was constant. There was a hyperbolic relationship between biosensor response/immobilized ascorbate oxidase activity and ascorbic acid concentration up to 500 μM, after which it was constant. The LB plot (Fig. 3) for immobilized ascorbate oxidase gave a K_m value of 200 μM for ascorbate, which is higher than that of free enzyme (180 μM) revealing decreased affinity of enzyme towards its substrate after immobilization [23], I_{max} for sensor was 50 mA. Table 1 summarizes comparison of present ascorbate biosensor with earlier biosensors. Only slight changes were observed in kinetic property of immobilized enzyme compared to native enzyme, which reveals that egg shell membrane proves a better and inert support for enzyme immobilization.

3.3. Evaluation of the biosensor

The following parameters were studied to evaluate the method.

3.3.1. Linearity

There was a linear relationship between current (mA) and ascorbic acid concentration ranging from 10 μM to 400 μM in reaction mixture, which is comparable to earlier reported biosensors [16,25,26] (Table 1).

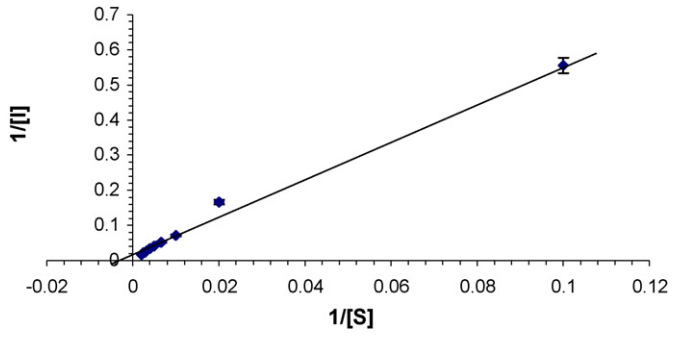

Fig. 3. Lineweaver–Burk plot for egg shell membrane bound bottle gourd fruit ascorbate oxidase.

Table 2
Analytical recovery of added ascorbate in serum, as measured by ascorbate biosensor based on egg shell membrane bound bottle gourd fruit ascorbate oxidase.

Ascorbate added (mg/dl)	Ascorbate found (mg/dl) Mean (n = 6)	% Recovery Mean ± SD
Nil	1.7	–
1.0	2.7	98.2 ± 3.1
2.0	3.6	96.7 ± 3.4

3.3.2. Detection limit

The detection limit of the present method was 1.0 μM, which is better/lower than earlier biosensors (2–4 μM) [16,26] (Table 1).

Table 3
Within and between assay coefficients of variation for determination of serum ascorbate in the serum samples, by ascorbate biosensor based on egg shell membrane bound bottle gourd fruit ascorbate oxidase.

n	Ascorbate (mg/dl) Mean ± SD	CV (%)
Within assay (6)		
1.32	1.27 ± 0.04	3.6
1.24		
1.22		
1.25		
1.33		
1.24		
Between assay (6)		
1.24	1.28 ± 0.05	4.49
1.35		
1.25		
1.24		
1.36		
1.24		

Table 4
Ascorbate level in sera of apparently healthy adults, as determined by ascorbate biosensor based on egg shell membrane bound bottle gourd fruit ascorbate oxidase.

Sex/age	Ascorbate (mg/dl) Mean ± SE[a]
F/24	0.61 ± 0.006
M/32	1.38 ± 0.009
M/30	1.52 ± 0.007
F/19	1.47 ± 0.006
M/23	1.30 ± 0.003
M/28	1.40 ± 0.005
M/35	1.32 ± 0.006
M/29	1.43 ± 0.003
M/21	1.23 ± 0.003
F/26	0.73 ± 0.230

[a] SE = standard error.

Table 5
Determination of ascorbic acid in various fruit juices by amperometric ascorbate biosensor based on egg shell membrane bound bottle gourd fruit ascorbate oxidase.

Source of fruit juice	Ascorbic acid content (mg/100 ml)		Literature values [2]
	By biosensor Mean ± SE	By DCPIP titration Mean ± SE	
Orange	53.3 ± 0.006	50.6 ± 0.004	30–50
Lemon	42.3 ± 0.008	41.8 ± 0.005	20–50
Apple	4.3 ± 0.005	4.7 ± 0.009	1.8–6.4
Grapefruit	47.7 ± 0.004	44.6 ± 0.006	24–40

3.3.3. Recovery

The analytic recovery of added ascorbate into serum (1.0 mg/dl and 2.0 mg/dl final concentration in reaction mixture) was 98.2 ± 3.1% and 96.7 ± 3.4% respectively (Table 2). These results are comparable to earlier reported o-phenylenediamine mediated ascorbate oxidase assay (>95%) [5] and biosensor based on carbon paste modified electrode (98.1–102.1%) [26].

3.3.4. Precision

To study the reproducibility and reliability of the present method, the ascorbate content in six serum samples was determined 6 times on single day (within batch) and again after storage at −20 °C for 1 week (between batch). The results showed that determinations were consistent and within and between batch coefficients of variation (CVs) were <3.6% and <4.49% respectively (Table 3), which indicate the reliability and consistency of the present method. The present CVs are comparable to earlier CVs by colorimetric method, HPLC (<5.0% within) [6] and also by o-phenylenediamine mediated ascorbate oxidase assay (<4.3% within and <6.7% between) [27].

3.3.5. Accuracy

To determine the accuracy of the method, the level of ascorbate in 10 serum samples was determined by standard DCPIP titrimetric method (x) and by the present method (y). The serum ascorbate values obtained by both the methods matched with each other with a good correlation (r = 0.993) (Fig. 4). Evaluation studies showed that the method was fairly reliable with high recovery and in agreement with the standard method.

3.4. Applications of the ascorbate biosensor

3.4.1. Serum ascorbate determination

The serum ascorbate level in apparently healthy adults as measured by present biosensor, ranged from 0.61 mg/dl to 1.52 mg/dl with a mean of 1.24 mg/dl (Table 4), which is in normal established range (0.4–1.5 mg/dl).

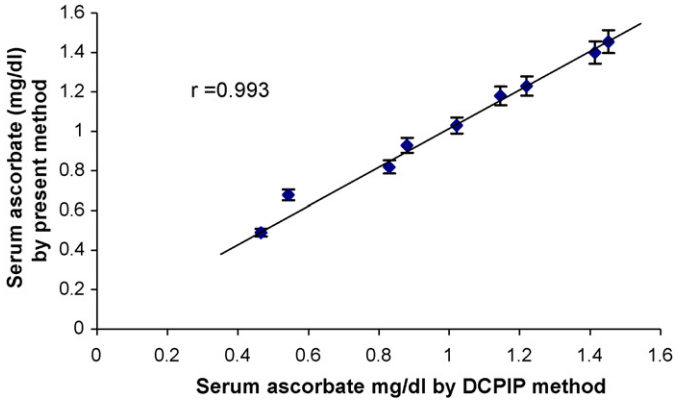

Fig. 4. Correlation between serum ascorbate values as determined by standard DCPIP titration method (x-axis) and present biosensor (y-axis) based on egg shell membrane bound bottle gourd ascorbate oxidase.

Table 6
Effect of various serum substances on response of ascorbate biosensor based on egg shell membrane bound bottle gourd fruit ascorbate oxidase.

Compound added (final concn. 0.1 mM)	% Relative response
None	100
Glucose	104
Fructose	102
Cysteine	112
Sucrose	103
Urea	97.5
Cholesterol	95.1
Starch	93.9
Sucrose	101
Sodium chloride	94.3
Tartaric acid	92.9
Lactose	95.5

3.4.2. Determination of ascorbate in fruits

The content of ascorbate in various fruits was determined by the present biosensor and the results are presented in Table 5. These values are in good agreement with earlier reports [16,26].

3.4.3. Determination of ascorbic acid in vitamin C tablets

Ascorbate content in vitamin C tablets as measured by the present sensor was 496.3 mg/tablet in lamcea and 192.7 mg/tablet in Becozyme C forte (multivitamin tablet). These results are also in good agreement with those by DCPIP method. This shows the reliability of the present method.

3.5. Interference study

Among the various serum metabolites tested, such as glucose, fructose, cysteine, sucrose, urea, cholesterol, starch, sucrose, sodium chloride, tartaric acid and lactose none caused significant interference in biosensor response (Table 6).

3.6. Reusability and storage

The enzyme electrode was reused for 200 times during the span of 90 days, when stored in 0.1 M sodium phosphate EDTA buffer pH 5.6 at 4 °C. This reusability and stability is higher than those of earlier biosensors [16,26,28].

4. Conclusion

An amperometric ascorbate biosensor was constructed using egg shell membrane bound ascorbate oxidase purified from bottle gourd fruit. This biosensor is simple, cost-effective and stable for 4 months with a detection limit of 1.0 μM. Our work also proves that egg shell membrane material is a promising support for immobilization of enzyme for construction of enzyme based biosensors.

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