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Determination of serum glucose with glucose oxidase immobilized onto affixed egg membrane

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A reusable strip of glucose oxidase (GOD) from *Aspergillus niger* was prepared by immobilizing the enzyme onto egg albumin membrane affixed on a plastic strip with a conjugation yield of 1.5 mg/cm^2 and 66% retention of their initial specific activity. The immobilized enzyme showed maximum activity at *p*H 7.0 when incubated at 37°C for 15 min. A method for discrete analysis of serum glucose was developed employing this strip. The minimum detection limit of the method was 5 mg/dL. Within and between assay coefficient of variation (CV) for serum were <5 and <6%. A good correlation (r=0.972) was found between glucose values obtained by commercial enzymic colorimetric method employing free GOD and the present method. No significant loss in the strip activity was observed after its 150 regular uses for a period of 40 days, when stored in reaction buffer at 4°C. The method has advantage over commercial enzymic colourimetric method that it provides reuse of glucose oxidase with ease.

Keywords: Glucose, Glucose oxidase, Immobilization, Egg membrane

IPC Code: C13K1/00, C07C

Determination of glucose in serum is required for the diagnosis and medical management of diabetes mellitus, hyper and hypo activity of thyroid, pituitary and adrenal gland, myxodema conditions interfering with glucose absorption. Enzymatic colorimetric method, employing glucose oxidase and peroxidase, though is comparatively simpler, sensitive, specific and requires only a colorimeter[1], yet the method is more expensive for routine use due to high cost of bulk quantity of the enzyme. The immobilization of the enzyme onto insoluble support permits its reuse and thus reduces the cost of procedure for a large number of clinical samples. Glucose oxidase from different sources has been immobilized onto various supports such as glass[2], alkylamine glass beads[3], nylon tubes[4], polyethene films[5], radiofrequency plasma modified poly(ether) urethane urea[6], polyprolene glass beads surface by covalent coupling[7], nafion membrane[8], graphite[9], polypyrrole/polytetrahydrofuran graft copolymer[10], electrode surface[11] and many more either alone or with peroxidase for determination of glucose[12]. All these supports used for immobilization of glucose oxidase, suffer from one drawback or the other such as high cost, limited availability, susceptibility to microbial attack or complicated procedure of their preparation. The present work was therefore carried out to immobilize GOD on a easily available and cheaper support i.e. egg membrane affixed on a plastic strip by a non-reactive fixative.

Experimental Procedure

Materials and Methods

Glucose oxidase from *Aspergillus niger*, 4-aminophenazone (Sigma USA), sodium nitrate, horseradish peroxidase (RZ= 2.0), dextrose (SRL, India) were used. Hen's eggs (white coloured) were purchased from local market.

Preparation of reusable strip of glucose oxidase

The rectangular strips of 15×1 cm size were cut from a plastic sheet (thickness: 0.5 mm). One end of each strip was made round with the help of a scissor.

Affixation of egg albumin membrane on plastic strip

One end of a plastic strip (15×1 cm) was made round with scissor and scratched with a sand paper on both sides up to a height of 2 cm. A thin layer of 0.1 mm of 'Araldite' fixative was spread uniformly on this scratched strip. Egg albumin was picked up from boiled white portion and affixed uniformly on this fixative layer. The strip was kept for 24 h at room temperature (25±3°C) for affixation of membrane.

Immobilization of glucose oxidase onto affixed egg albumin membrane

Activation of affixed egg albumin membrane was carried out as described[13]. The end of plastic strip containing affixed egg membrane was dipped into a test tube containing 5 mL glutaraldehyde solution (2.5%) in 0.1 M sodium phosphate buffer (*p*H 7.0). The egg membrane was allowed to get activated for about 2 h at room temperature, with occasional stirring. The excess of glutaraldehyde solution was discarded and affixed egg membrane was washed with 0.1 M sodium phosphate buffer (*p*H 7.0) 8-10 times until the *p*H of discard was 7.0. The end of the strip containing affixed egg albumin membrane was dipped into 2.0 mL glucose oxidase (1.0 mg/mL) in 0.1 M sodium phosphate buffer (*p*H 7.0) in 15 mL test tube (Size: O.D × length = 2.5×5.7 cm). It was kept for 48 h at 4°C with occasional shaking. The strip was taken off from enzyme solution, which was tested for enzyme activity. The strip was dipped into distilled water 5-6 times until no activity of enzyme was detected in the consequent washing. The enzyme bound to membrane was measured by determining the protein in the enzyme preparation before and after immobilization[14].

Assay of strip bound glucose oxidase

The plastic strip containing immobilized glucose oxidase onto affixed egg membrane was termed as 'enzyme strip'. The assay was carried out in a 15 mL test tube wrapped with black paper. To 1.9 mL 0.1 M sodium phosphate buffer (*p*H 7.0), the enzyme strip was inserted in such a manner that its end containing immobilized enzyme is dipped in the reaction buffer. After preincubation at 37°C for 5 min, the reaction was started by adding 0.1 mL glucose solution (1 mg/mL). After incubating the reaction mixture at 37°C for 20 min under gentle and continuous stirring in water bath shaker, the enzyme strip was taken off and 1.0 mL colour reagent (4-aminophenazone, 18 mg; phenol, 36 mg and horseradish peroxidase, 1 mg in 100 mL 0.1 M sodium phosphate buffer, *p*H 7.0) was added and kept at room temperature for 15 min. The reaction mixture was transferred to a cuvette and A_{520} was read in Spectronic-20 (Million & Roy, USA) against control. The control was prepared in the same manner except that strip had only affixed egg membrane. Both the enzyme and control strips were washed in distilled water after the assay and stored in 0.05 M sodium phosphate buffer (*p*H 7.0) at 4°C until use (Fig. 1A, 1B).

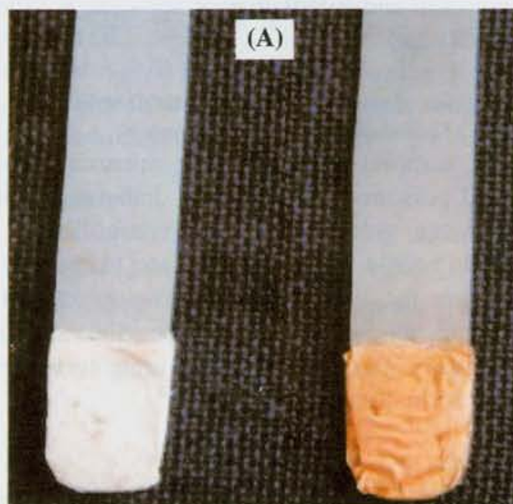

Fig. 1A—Control: A plastic strip containing an egg albumin membrane affixed on its one end
Test: A plastic strip containing affixed egg albumin membrane coupled to glucose oxidase through glutaraldehyde
Fig. 1B—Test tube showing the reaction mixture of control and test after completion of assay of glucose oxidase. Control contains 1.9 mL 0.1 M sodium phosphate buffer *p*H 7.0+ 0.1 mL glucose (1 mg/mL) + and plastic strip containing affixed egg albumin membrane. Test contains 1.9 mL 0.1 M sodium phosphate buffer *p*H 7.0 + 0.1 mL glucose (1 mg/mL) + plastic strip containing affixed egg albumin membrane immobilized to glucose oxidase. After incubation at 37°C for 15 min, 1 mL colour reagent (18 mg 4-aminophenazone, 36 mg phenol and 1 mg horseradish peroxidase per 100 mL 0.1 M sodium phosphate buffer *p*H 7.0) added to reaction mixture in both test tubes and kept at room temperature for 15 min to develop the colour.

Kinetic properties of strip bound GOD

Following kinetic properties of immobilized enzymes were studied-optimum *p*H, incubation temperature, time of incubation, effect of glucose

concentration and calculation of apparent K_m and V_{max} (from L-B plot)

Determination of serum glucose with 'GOD strip'

Collection of blood and preparation of serum

Blood samples (2 mL) from apparently healthy adult and persons suffering from diabetes mellitus of different age groups and sex were collected using sterilized needle and syringe and kept at room temperature for 1 h. After centrifuging at 2000 rpm for 5 min at room temperature, the supernatant (serum) was collected and diluted in reaction buffer in 1:1 ratio at the time of use.

Determination of serum glucose

It was carried out in the same manner as described for assay of strip bound GOD under optimum assay conditions except that the glucose solution was replaced by diluted serum (50 μL serum sample +50 μL reaction buffer). The glucose concentration in serum was extrapolated from standard curve between glucose concentration ranging 20 to 270 mg/dL and A_{520} prepared under optimal assay condition.

Reuse and storage of enzyme strip

To reuse the enzyme strip, its end containing immobilized enzyme was washed off with distilled water 4-5 times prior to use in next assay. The enzyme strip was stored at 4°C in reaction buffer when not in use.

Results and Discussion

Commercially available GOD from *Aspergillus niger* was immobilized through glutaraldehyde coupling onto egg albumin membrane affixed on a plastic strip by non-reactive fixative with 66% retention of initial activity of free enzyme and 1.5 mg/cm^2 conjugation yield (Table 1). The enzyme was immobilized covalently through glutaraldehyde between $-NH_2$ groups on the surface of enzyme and affixed egg albumin membrane, due to which it was used repeatedly.

Kinetic properties of immobilized GOD

Table 2 summarizes the comparison of kinetic properties of free and egg membrane bound GOD. Compared to free enzyme, the membrane bound GOD showed an increase in optimum *p*H from 5.5 to 7.0. A similar increase in *p*H of GOD (*p*H, 5.5-7.0) has been reported for enzyme sensor based on GOD immobilized on Sigma glass-supported aminopropyl[15]. An increase in K_m value for glucose from 6.2 to 12.5 mM V_{max} from 0.12 to 0.98 nmol/H_2O_2/min and energy of activation (E_a) from 2.05 to 5.34 Kcal/mole but decrease in time of incubation from 20 to 15 min was observed after immobilization. A decrease in incubation time (20 to 10 min) has also been reported for enzyme sensor of glucose[15]. However, there was no change in incubation temperature for maximum activity (37°C) after immobilization. The change in kinetic properties of immobilized enzyme might be due to change in the enzyme conformation, steric hindrance, micro-environmental effect and bulk and diffusional effects after immobilization[16].

Method for determination of serum glucose

A simple, sensitive and specific method for discrete analysis of glucose in serum was developed employing strip bound GOD. The method is based on the measurement of H_2O_2 generated from serum glucose by immobilized glucose oxidase by a colour reaction consisting of 4-aminophenazone, *p*-hydroxy benzoic acid and peroxidase as chromogenic system. The method has the advantage that it provides the efficient reuse of GOD with ease. Further, the method provides an easily available cheaper support for immobilization of GOD and is free from possible

Table 1—Immobilization of glucose oxidase from *Aspergillus niger* on to egg membrane affixed on a plastic strip

Enzyme added to membrane (mg proteins)	4.6
Enzyme coupled to membrane (mg proteins)	3.0
Enzyme Unit added (nmolH_2O_2/mL)	740
Conjugation Yield (mg/area in cm^2)	1.5
% Enzyme coupled	65.5
% Retention of Sp activity	66

Table 2—Kinetic parameters of free and immobilized glucose oxidase from *Aspergillus niger* onto egg albumin membrane affixed on a plastic strip

Parameter	Free	Immobilized
Optimum *p*H	5.5	7.0
Temperature for maximum activity	37°C	37°C
E_a (K Cal./mole)	2.05	5.34
Time of incubation (min)	20	15
Saturating conc. of glucose (mM)	10	20
Km for glucose (mM)	6.2	12.5
Vmax (μmol/min)	0.120	0.98

Data are the mean of three replicates

interference of immobilized enzyme system by the accumulation of product of colour reagent by its repeated use. Following analytical parameters were studied to evaluate the method.

Linearity

A linear relationship was found between A_{520} versus glucose concentration ranging from 20-270 mg/dL in reaction mixture (Fig. 2), which is similar to that by co-immobilized glucose oxidase and peroxidase onto arylamine glass beads affixed on a plastic strip (20-100 mg/dL)[15] but higher then that by enzymic sensor (5-20 mg/dL)[16].

Detection limit

The lower detection limit of the method is 5 mg/dL, which is similar to that by co-immobilized glucose oxidase and peroxidase onto arylamine glass beads affixed on a plastic strip (5 mg/dL)[15], but higher than that by GOD and horse radish peroxidase immobilized individually onto alkylamine glass beads (3.6 mg/dL)[3] and lower than that direct fluorimetric determination (8.5 mg/dL)[7]. The upper detection limit of the method is 270 mg/dL. As the serum is diluted in 1:1 ratio before measurement of sugar, the method is able to measure glucose in serum up to 540 mg/dL.

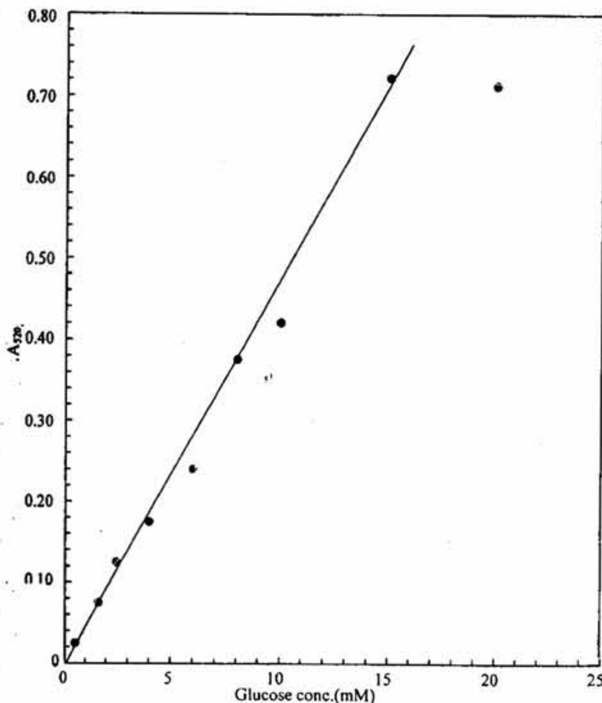

Fig. 2—Standard curve between glucose conc. versus A_{520} by the present method using a egg albumin membrane bound glucose oxidase affixed on a plastic strip. The reaction mixture containing 1.9 mL 0.1 M sodium phosphate buffer *p*H 7.0 + 0.1 mL glucose solution and plastic strip containing affixed egg albumin membrane bound to glucose oxidase were incubated at 37°C for 15 min. The strip was taken off and 1.0 mL colour reagent was added to reaction mixture and kept at room temperature for 15 min. A_{520} was read.

Analytical recovery

The mean analytical recovery of added solid glucose (1 mg/mL) in serum samples was 82.0%±0.70 (mean ± S.D.; n=6) which is comparable to that of using co-immobilized glucose oxidase and peroxidase onto arylamine glass beads affixed on a plastic strip (90.3%)[15].

Precision

To check the reproducibility and reliability of the method, glucose content was measured in the same serum sample six times in one run (within batch) and same serum sample after one week of storage at -20° C (between batch). The within and between day coefficient of variation (CV) for serum glucose determination were <5.0 and <6.0%, respectively, lower to that by co-immobilized glucose oxidase and peroxidase onto arylamine glass beads affixed on a plastic strip (<5.6% for within batch and <10% for between batch)[15] indicating good reproducibility and reliability of the method.

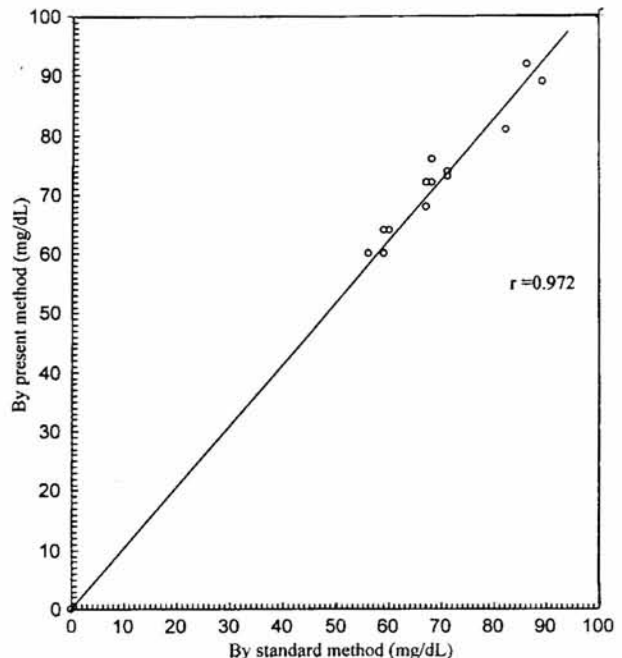

Fig. 3—Correlation between serum glucose as determined by the commercial enzymic colourimetric method (x) and the present method using egg albumin membrane membrane bound glucose oxidase affixed on a plastic strip (y).

Table 3—A comparison of serum glucose by commercial enzymic colourimetric method and the present method using egg albumin membrane bound glucose oxidase affixed on a plastic strip

Sample No.	Commercial enzymic colourimetric method (mg/dL)	Present method (mg/dL)
1	56	60
2	60	60
3	60	64
4	62	64
5	68	68
6	70	74
7	70	74
8	68	72
9	69	72
10	82	82
11	87	92
12	89	89

Data are the mean of three replicates

Accuracy

To test the accuracy of the method, the glucose value of serum samples obtained from normal persons by commercial enzyme colorimetric method employing free GOD and the present method was compared (Table 3). The correlation coefficient (r) was 0.972, indicating high accuracy of the method (Fig. 3).

Determination of serum glucose

The glucose value in serum samples obtained from normal and diabetic individuals of various age groups and sex was measured by the present method, which ranged from 60 to 90 mg/dL with a mean of 75 mg/dL (n=20) and 150 to 230 mg/dL with a mean of 178 mg/dL (n=20), respectively.

Reusability and storage stability of GOD strip

The immobilized enzyme strip did not show any considerable loss of its activity after its regular use for over a period of 40 days when stored in 0.1 M reaction buffer at 4°C. During this period the enzyme strip was used for 150 times.

In conclusion, a reusable strip of GOD has been prepared for serum glucose determination. The strip has the advantage over commercial enzymic method, as it provides the reuse of GOD with ease.

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