International Journal of Biological Macromolecules 47 (2010) 691–695

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Determination of serum triglyceride by enzyme electrode using covalently immobilized enzyme on egg shell membrane

Jagriti Narang, Minakshi, Manu Bhambi, C.S. Pundir*

Biochemistry Research Laboratory, Department of Biochemistry, M D University, Rohtak 124001, India

ARTICLE INFO

Article history:
Received 27 July 2010
Received in revised form 30 August 2010
Accepted 1 September 2010
Available online 9 September 2010

Keywords:
TG
Biosensor lipase
GK
GPO
Egg shell membrane

ABSTRACT

A mixture of commercial lipase, glycerol kinase and glycerol-3-phosphate oxidase was co-immobilized onto egg shell membrane through covalent coupling. A method is described for fabrication of a triglyceride (TG) biosensor using egg shell membrane bound enzymes. The biosensor measured current, i.e. flow of electrons generated from H_2O_2, maximally when polarized at 0.4 V. The biosensor showed optimum response within 10 sec at pH 7.0 and 35 °C. The current was in proportion to concentration of TG in the range 0.56–2.25 mM. An amperometric method was developed for determination of TG employing this enzyme electrode. The minimum detection limit of the method was 0.28 mM. The analytic recovery of added TG was 95.00% and 96.50%. Within batch and between batch coefficients of variations (CV) were <2.14% and <3.48% respectively. A good correlation (r = 0.985) was obtained between serum TG level by standard enzymic colorimetric method and the present method. Serum substances such as urea, uric acid, glucose, cholesterol, ascorbic acid and pyruvic acid had no interference. The enzyme electrode was used 200 times over a period of 70 days without any considerable loss of activity, when stored at 4 °C.

1. Introduction

Triglycerides (TG) are transesterification product of fatty acids and glycerol and engaged in the transportation of fats. TG level in serum of a healthy person is in the range 150–190 mg/dl, When TG level is higher than 200 mg/dl, two ranges of hypertriglyceridimia arise: high (200–499 mg/dl) and severe high level (>500 mg/dl). Elevated triglyceride levels are associated with coronary heart disease (CAD), atherosclerosis and hypolipoprotenimia [1]. Current analytical methods for determination of serum triglycerides are tedious, time consuming and require expensive reagents, equipment and skilled person to operate [2–6]. Immobilized enzyme based biosensors are comparatively more simple, sensitive and rapid. There are few enzyme based TG biosensors, such as amperometric biosensor based on collagen membrane [7] amperometric biosensor using dissolved oxygen electrode [8,9], potentiometric biosensor using porous silicon [10], amperometric biosensor using prussian blue modified screen printed electrode [11]. Other amperometric triglyceride biosensors are based on artificial membrane bound enzymes such as collagen, [7] CA [14] and PVC [12] membranes. The collagen membrane had a problem of time consuming (3 days) preparation, long response time and low storage stability. Although CA and PVC membranes were better than collagen membrane, these were more elastic, non-fragile and chemical resistant, all these had low permeability and conductivity and a major problem of desorption or leaching of enzymes during washing. We have reported a new technique of covalent immobilization of enzymes onto egg shell membrane which is expected to overcome this problem. Further an egg shell membrane had excellent gas and water permeability, biocompatible, low cost and stable [13]. The present report describes the construction of a triglyceride biosensor using egg shell membrane bound enzymes through covalent coupling.

2. Experimental

2.1. Reagents and materials

Triton X-100 from Sigma Chemicals Co, USA; 3, 5-dichloro-2-hydroxy benzene sulphonic acid (DHBS) from E-Merk Germany, triolein and ATP from SISCO Research Laboratory Pvt. Ltd., Mumbai and kit for enzymic colorimetric method (Enzo kit) for TG from Erba Transasia, Daman, India were used. All other chemicals were of AR grade.

2.2. Preparation of mixture of lipase, GK and GPO

To prepare mixture of lipase, glycerol kinase (GK) and glycerol-3-phosphate oxidase (GPO), 3.0 mg of Reagent 1 of Enzo kit for TG consisting of lipase (110U), GK (80U), GPO (500U) and

* Corresponding author at: Department of Biochemistry, M D University, Rohtak 124001, Haryana, India. Tel.: +91 9416492413.
E-mail addresses: pundircs@rediffmail.com, pundircs@rgmail.com (C.S. Pundir).

0141-8130/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijbiomac.2010.09.001

4-aminoantipyrine was dissolved in 1 ml of 0.02 M sodium phosphate buffer pH 7.0 and loaded on a Sephadex G-100 gel column (24 cm × 1 cm) preequilibrated with 0.02 M sodium phosphate buffer pH 7.0. The column was run in the same buffer at a flow rate of 0.5 ml/min. The fractions (2 ml each) were collected and tested for protein by Lowry method. The fractions showing presence of protein were pooled and treated as mixture of lipase, GK and GPO. The mixture was tested for combined activity of lipase, GK and GPO as given below.

2.3. Preparation of triolein solution

Triolein was used as a substrate for lipase, GK and GPO. Triolein emulsion was prepared as described [15]. Solutions of different concentrations of triolein ranging from 0.1 to 20 mM were prepared in 0.1 M sodium phosphate buffer (pH 7.0) and stored at 4 °C until use.

2.4. Assay of mixture of free lipase, GK and GPO

The assay of mixture of free lipase, GK and GPO was carried out in a 15 ml conical flask wrapped in a black paper [2]. The reaction mixture consisted of 0.54 μmol $MgCl_2$, 0.63 μmol ATP, 1.0 μmol potassium ferrocyanide, 0.27 μmol 4-aminophenazone, and 1.53 μmol DHBS, 1.0 μmol. of Triton X-100/liter of 0.1 M sodium phosphate buffer, pH 7.0. To 900 μl reaction mixture, 50 μl of mixture of enzymes was added and pre-incubated at 37 °C for 5 min. The reaction was started by adding 50 μl of 22.6 mM triolein. After incubation at 37 °C for 15 min, A_{510} was read. H_2O_2 generated in reaction was calculated from standard curve between A_{510} versus H_2O_2 concentration. The unit activity of mixture of enzyme was expressed as 1 μmol H_2O_2/min/ml.

2.5. Preparation and activation of egg shell membrane

An egg shell membrane was removed carefully from a boiled egg after removing its hard covering and yolk. It was washed thoroughly with distilled water for removal of albumin. The membrane was cut into rectangular pieces of 5 cm × 2 cm dimension. These pieces were washed again with distilled water. The membrane was activated by immersing it into a layer of reagent A consisting 50 mg of nickel chloride dissolved in 10 ml liquid ammonia, in a petridish. After keeping it at room temperature for 5 h, the membrane was removed and washed thoroughly with distilled water to remove excess of reagent A. The membrane was treated with reagent B consisting 2.5% glutaraldehyde solution in 0.1 M sodium phosphate buffer, pH 7.0, for 2 h at ambient temperature. The membrane was washed thoroughly with distilled water to remove excess of glutaraldehyde [16].

2.6. Co-immobilization of lipase, GK and GPO onto egg shell membrane

Lipase, GK and GPO were co-immobilized on egg shell membrane through glutaraldehyde coupling. The mixture of enzymes (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 sodium phosphate buffer, pH 7.0) to remove unbound enzymes [16]. The co-immobilization occurred by coupling of $-NH_2$ groups on surface of enzyme's with amino groups introduced on egg membrane by glutaraldehyde. Fig. 1 depicts the chemical reaction involved in immobilization of enzyme(s) on egg shell membrane.

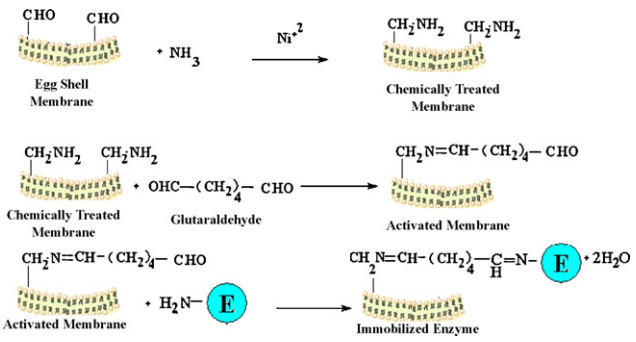

Fig. 1. Scheme of immobilization of enzyme on egg shell membrane.

2.7. Construction of amperometric TG biosensor and response measurement

An amperometric biosensor for measurement of TG was constructed by mounting egg shell membrane bound to lipase, GK and GPO onto Pt electrode with a parafilm to use it as a working electrode. This working electrode along with Ag/AgCl as reference and Cu wire as auxiliary electrode were dipped into 4 ml reaction mixture [1.0 ml triolein and 3 ml 0.1 M sodium phosphate buffer pH 7.0] and connected through electrometer (Make: Keithley Japan, Model 6517A/E). The electrodes were polarized at different potential (in volts) and current (mA) generated was measured in electrometer. The current was also measured at varying concentration of triolein. The principle of working of the biosensor was as follows. As soon as electrode system triolein was dipped into the reaction mixture, it converted triolein into dihydroxyacetone phosphate and H_2O_2. H_2O_2 under high potential applied through electrometer was splitted into $2H^+ + O_2 + 2e^-$. The electrons thus generated from H_2O_2, were passed from solution to Pt electrode through pores of egg shell membrane [10]. The reactions involved were as follows:

$$\text{triglyceride} \xrightarrow{\text{lipase}} \text{glycerol} + \text{fatty acids}$$

$$\text{glycerol} + \text{ATP} \xrightarrow[MgCl_2]{GK} \alpha\text{-glycerol- 3-phosphate} + \text{ADP}$$

$$\text{glycerol-3-phosphate} + O_2 \xrightarrow{GPO} \text{dihydroxyacetone phosphate} + H_2O_2$$

$$H_2O_2 \xrightarrow{0.4V} 2H^+ + O_2 + 2e^-$$

where GK = glycerol kinase and GPO = glycerol-3-phosphate oxidase.

2.8. Optimization of working conditions of TG biosensor

Various kinetic properties of egg shell membrane bound enzymes mounted on Pt electrode such as optimum pH, incubation temperature, time for maximum response and effect of substrate (triolein) concentration were studied amperometrically in order to optimize the working conditions of the biosensor.

2.9. Amperometric determination of TG in serum

Blood samples (1 ml each) from apparently healthy male and female (25 each) and persons suffering from hypertriglyceridemia of various categories such as peripheral vascular disease and pancreatitis were collected from local Pt BDS PGIMS, Rohtak hospital and centrifuged at 5000 rpm for 5 min and their supernatant (serum) was collected. TG content in serum was determined by the present biosensor in same manner as described above for

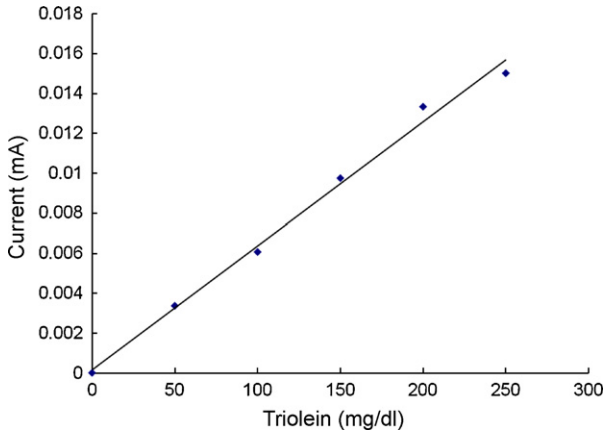

Fig. 2. Standard curve for triolein concentration by TG biosensor based on egg shell membrane bound lipase, GK and GPO at 0.4 V. Optimal working conditions were used except for triolein concentration which was varied as given above.

its response measurement, under its optimal working conditions except that triolein was replaced by serum. The current (mA) was measured and the amount of TG in serum was extrapolated from standard curve between triolein concentrations and current (in mA) prepared under optimal working conditions (Fig. 2).

2.10. Evaluation of TG biosensor

The biosensor was evaluated by studying analytic recover, precision and correlation. The effect of various possible interfering substances found in blood such as uric acid, cholesterol, ascorbic acid, bilirubin, glucose, pyruate and glutathione was also studied at their physiological concentrations.

3. Results and discussion

3.1. Co-immobilization of lipase, glycerol kinase and glycerol-3-phosphate oxidase on to egg shell membrane

A mixture of commercial lipase, glycerol kinase and glycerol-3-phosphate oxidase was co-immobilized covalently onto egg shell membrane through glutaraldehyde coupling [16]. The treatment of egg shell membrane with liquid ammonia in presence $NiCl_2$ resulted into introduction of $-NH_2$ groups on its surface. The glutaraldehyde worked as a cross-linking agent between lipase, GK and GPO and activated egg shell membrane. One –CHO group of glutaraldehyde linked covalently to $-NH_2$ groups of egg membrane, while other –CHO group was covalently linked to $-NH_2$ groups on the surface of lipase, GK and GPO. This provided more stable bioconjugate of enzyme and support than attained by physical adsorption [16].

3.2. Construction of TG biosensor

A method is described for preparation of an amperometric TG biosensor employing egg shell membrane bound covalently to lipase, GK and GPO. The TG biosensor showed good response in the form of current (mA). As the current generated was optimum at +0.4 V, it was selected for standardization of working conditions of biosensor.

3.3. Optimization of TG biosensor

3.3.1. Optimum pH

To determine optimum pH of the biosensor, pH of reaction buffer was varied from pH 6.0 to 8.0 at an interval of pH 0.5, using 0.05 M sodium phosphate buffer. The biosensor response was increased as the pH was increased up to pH 7.0, after which it was decreased (Fig. 3) The optimum pH of the present electrode (pH 7.0) was lower than amperometric sensor based on Prussian blue modified screen printed electrodes (pH 8.0) [11] and Pt electrode mounted with PVC membrane bound enzymes (pH 7.5) [12] but slightly higher than biosensor based on CA membrane bound enzymes (pH 6.5) [14].

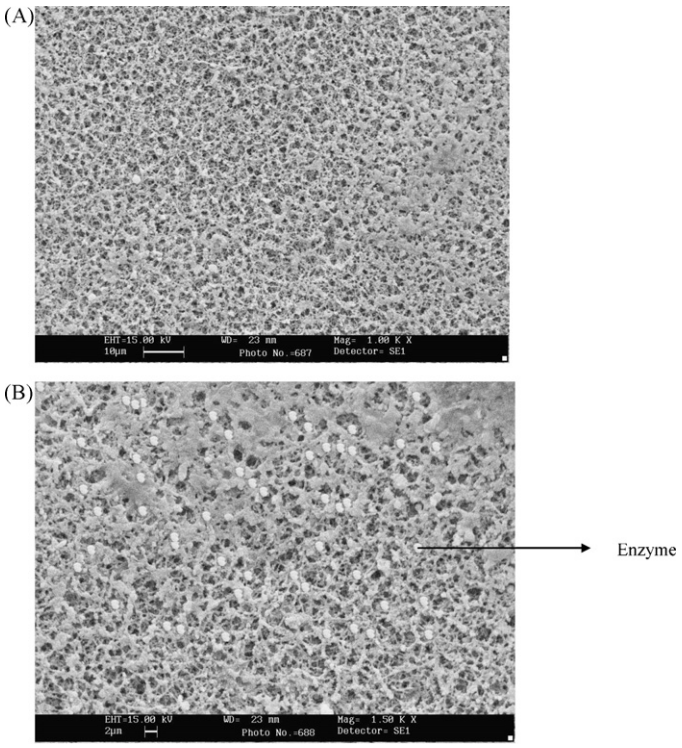

Fig. 3. SEM pictures of (A) a egg shell membrane and (B) enzymes immobilized on egg shell membrane.

3.3.2. Optimum temperature

The biosensor response was evaluated at different incubation temperatures from 25 °C to 40 °C at an interval of 5 °C. The biosensor response was increased as incubation temperature was increased up to 35 °C, thereafter it declined (Fig. 4). Hence all the subsequent assays were carried out at 35 °C. This is similar to that biosen-

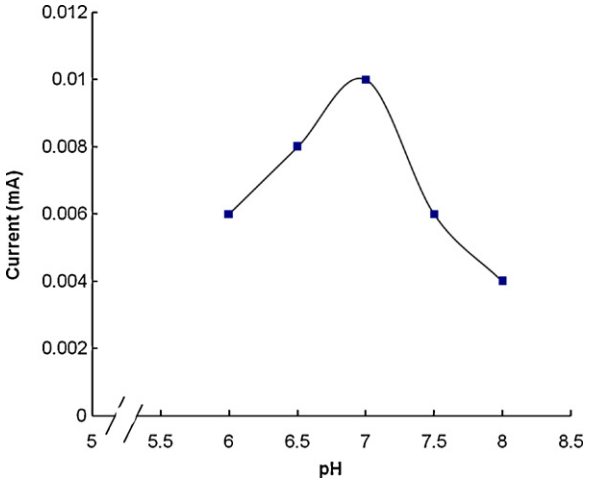

Fig. 4. Effect of pH on response of TG biosensor based on egg shell membrane bound Lipase, GK and GPO at 0.4 V in 2.25 mM triolein in 0.01 M sodium phosphate buffer whose pH was varied between pH 6.0 and 8.0 at an interval of pH 0.5.

Table 1
A comparison of kinetic parameters of free and co-immobilized lipase, glycerol kinase and glycerol-3-phosphate oxidase on CA membrane and egg shell membrane.

Parameter	Mixture of free enzymes	Co-immobilized on CA membrane [14]	Co-immobilized on PVC membrane [12]	Co-immobilized on egg shell membrane
Optimum pH	7.0	6.5	7.5	7.0
Optimum temp	37 °C	25 °C	35 °C	35 °C
Thermal stability (at 70 °C for 15 min)	30%	43%	45%	55%
Time for linearity	15 min	40 s	30 s	30 s
Km (for triolein)	21.1 mM	8.76 mM	6.0 mM	6.0 mM
Vmax (for triolein)	19.6 nmoles/min	0.83 nmoles/min	1.15 nmoles/min	1.15 nmoles/min
Stability at 4 °C	42%	50% loss in 25 days	50% loss in 40 days during regular use for 6 months (loss of initial activity)	50% loss in 70 days

sor based on PVC membrane [12] but higher than CA membrane bound enzyme sensor (25 °C) [14]. This optimum temperature was lower than that based on oxygen meter biosensor (39.5 °C) [9] and amperometric biosensor based on collagen membrane (37 °C) [7].

3.3.3. Effect of substrate concentration

To study the effects of substrate concentration on biosensor response, the concentration of triolein was varied from 0.5 mM to 2.1 mM. Km for triolein as calculated from Lineweaver Burk plot (Fig. 5), was 6.0 mM, which is lower than that of CA membrane bound enzymes (10 mM) [14] but similar to biosensor based on PVC membrane [12] A comparison of various kinetic properties of egg shell membrane bound enzymes with those of free and CA and PVC membrane bound lipase, GK and GPO is summarized in Table 1.

3.4. Determination of TG with biosensor

An amperometric method for determination of TG was developed using the present biosensor. The method has the advantage over earlier methods that it employ covalently bound enzymes on naturally occurring cheaper support (egg membrane), which provides stability of immobilized enzyme and better flow of electrons compared to previous membrane electrodes [12,14].

3.5. Evaluation of TG biosensor

The following criteria were studied to evaluate the performance of this biosensor:

3.5.1. Linearity

There was a linear relationship between current (mA) and triolein concentration ranging from 0.56 mM to 2.25 mM in reaction mixture, which is similar to that biosensor based on PVC membrane

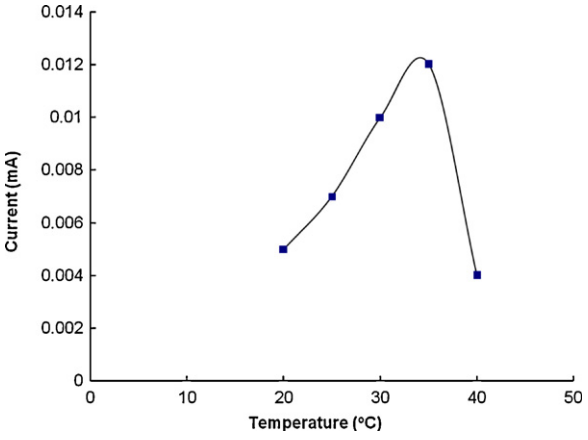

Fig. 5. Effect of incubation temperature on the TG biosensor based on egg shell membrane bound Lipase, GK and GPO at 0.4 V at 2.25 mM triolein concentration.

[12] but lower than that for dissolved oxygen (DO) metric biosensor [5–20 mM] [9] and comparable to enzymic sensor (0.3–2.5 mM) [17] porous silicon based biosensor (0.2–2.1 mM) [10] and CA membrane bound enzyme sensor (0.2–3.5 mM) [14]

3.5.2. Detection limit

The minimum detection limit of the method was 0.28 mM, which is similar to CA membrane based enzyme electrode (0.2 mM) [14] but lower than DO metric biosensor (0.35 mM) [9], potentiometric biosensor (0.42 mM) [10], amperometric biosensor (0.32 mM) [7] and PVC membrane bound enzyme sensor (0.1 mM) [12].

3.5.3. Recovery

The % recovery of added triolein in serum (10 mg/dl and 20 mg/dl) as determined by the present biosensor, was 95.00% and 96.50% respectively (Table 2), which is higher than that of CA membrane based biosensor (89%) [14] and with prussian blue modified screen printed electrode (81%) [11] but lower than amperometric enzyme biosensor (98%) [7].

3.5.4. Precision

Within and between batch coefficient of variation (CV) for serum TG determination by the present biosensor were <2.14% and <3.48% respectively (Table 3), which is comparable to those by oxygen meter based biosensor (<2.18% and <1.7%) [9] but lower than those amperometric enzyme biosensors (6.2 and 7.4%) [7] (<2.8% and <4.9%), [11] <2.53% and <3.21% [12] (Table 3) and CA membrane bound enzyme biosensor (<8%and <4%) [14].

3.5.5. Accuracy

To study the accuracy of the present method, TG level in 20 serum samples were determined by both the commercial enzymic colorimetric method (x) and the present method (y). The values obtained by both the methods matched with each other with a good correlation ($r = 0.985$) (Fig. 6). This r value is better than those by amperometric DO metric biosensor ($r = 0.97$) [9], CA membrane bound enzyme sensor (0.91) [14] and PVC membrane bound enzyme sensor ($r = 0.91$) [12] but comparable to that by other amperometric biosensor ($r = 0.993$) [7]

Table 2
Analytical recovery of added triglyceride in serum samples determined by TG biosensor based on egg shell membrane bound lipase, glycerol kinase and glycerol-3-phosphate oxidase.

Triglyceride added (mg/dl)	Triglyceride found (mg/dl)	% recovery
–	180	–
20	199.2	96.00
50	227.3	94.60

Table 3
Within and between batch assay coefficient of variation (CV) for determination of serum triglycerides by TG biosensor based on egg shell membrane bound lipase, glycerol kinase and glycerol-3-phosphate oxidase.

n	Triglycerides (mg/dl)	CV (%)
Within assay (5)		
111.89	110.90 ± 2.38	2.14
109.23		
108.23		
114.35		
110.8		
Between assay (5)		
115.8		
120.5	119.38 ± 4.16	3.48
121.9		
114.4		
124.3		

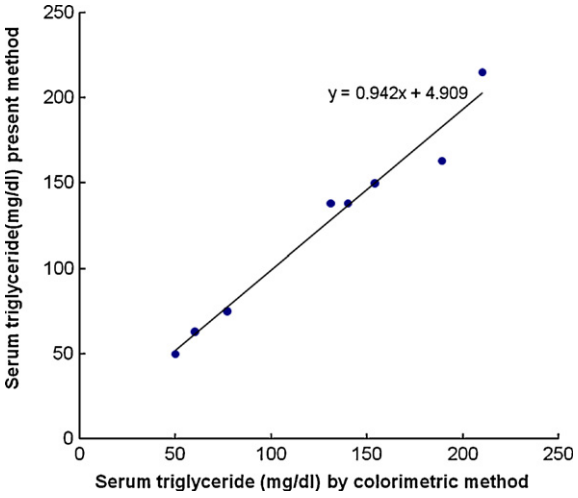

Fig. 6. Correlation (0.985) between serum triglyceride (mg/dl) determinations by Standard enzymic colorimetric method (*x*) and present amperometric method (*y*).

3.5.6. Interference study

Among the various serum substances tested such as urea, uric acid, glucose, cholesterol, ascorbic acid and pyruvic acid, at their physiological concentration, none had any practical interference (Table 4)

3.6. Determination of serum TG

TG Serum level in apparently healthy adults as measured by the present biosensor ranged from 50 to 180 mg/dl which is in established normal range (40–190 mg/dl). The TG level in the serum of persons suffering from hypertriglyceridemia was in the range 200–499 mg/dl, similar to earlier reports [12].

Table 4
Effect of interfering substances for determination of serum triglycerides by TG biosensor based on egg shell membrane bound lipase, glycerol kinase and glycerol-3-phosphate oxidase.

Substances	Relative activity (%)
Glucose	100
Uric acid	96
Cholesterol	105
Ascorbic acid	100
Bilirubin	100
Urea	97
Pyruate	100

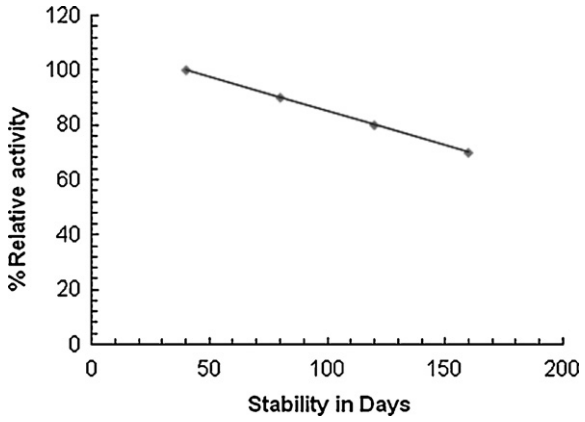

Fig. 7. Effect of storage on the response of biosensor based on egg shell membrane bound Lipase, GK and GPO at 0.4 V in 0.1 M sodium phosphate buffer, pH 7.5 at 4 °C.

3.7. Reusability and storage

The enzyme electrode was reused 200 times over a period of 70 days when stored at 4–8 °C in desiccators (Fig. 7). This reusability of this biosensor was better than DO metric enzyme sensor [9] amperometric biosensor [7] PVC membrane bound enzyme sensor [12] and CA membrane bound enzyme biosensor [14].

4. Conclusion

A mixture of lipase, glycerol kinase and glycerol-3-phosphate oxidase was immobilized covalently onto egg shell membrane. This enzyme membrane conjugate was employed for construction of an amperometric TG biosensor for determination of TG in serum. This biosensor has advantage over previous membrane based enzyme sensor that it has no problem of leaching of enzymes from membrane support and provides better flow of electrons, i.e. current.

Acknowledgment

The present work was supported by a grant to one of the author (CSP) from Department of Biotechnology, Govt. of India New Delhi.

References

[1] M. Haim, M. Benderly, D. Brunner, S. Behar, E. Graff, H. Reicher-Reiss, U. Goldbourt, Elevated Serum Triglyceride Levels and Long-Mortality in Term Patients With Coronary Heart Disease, BIP, 1999.
[2] P. Fossati, L. Prencipe, Clin. Chem. 28, (1982) 2077–2080.
[3] J.E. Voysey, D.C. Wilton, Clin. Chem. 40 (1994) 14–17.
[4] M. Okazaki, N. Komoriya, H. Tomoike, N. Inowe, S. Itoh, S. Hoosaki, J. Chromatogr. B: Biomed. Sci. Appl. 709 (1998) 179–187.
[5] E. Van Handel, D.B. Zilversmit, J. Lab. Clin. Med. 50 (1957) 152–157.
[6] I. Bjorkhem, R. Blomstrand, L. Svensson, Clin. Chim. Acta 71 (1976) 191–198.
[7] H. Winartasaputra, S.S. Kutan, G.C. Cuilbault, Anal. Chim. 54 (1982) 1987–1990.
[8] T.A. Kelly, G.D. Kristan, Analyst 109 (1984) 453–456.
[9] M. Bhambi, C.S. Minakshi, Pundir, Sens. Transd. 67 (2006) 561–567.
[10] R. Reddy, R.C. Anju, B. Enakshi, Biosens. Bioelectron. 16 (2001) 313–317.
[11] I. Ben Rejeb, F. Arduini, A. Amine, M. Gargouri, G. Palleschi, Anal. Chim. Acta 594 (2007) 1–8.
[12] J. Narang, M. Bhambi, C.S. Minakshi, Pundir, Anal. Lett. 43 (2010) 1–11.
[13] G. Zang, D. Liu, S. Shuang, M.F. Choi, Sens. Actuators 114 (2006) 936–942.
[14] Minakshi, C.S. Pundir, Sens. Actuats B: Chem. 133 (2008) 251–255.
[15] N.P. Ehle, C.M. Schotz, J. Lipid Res. 17 (1976) 536–546.
[16] C.S. Pundir, M. Bhambi, N. Chauhan, Talanta 77 (2009) 1688–1693.
[17] S.P. Ivashkevyeh, V.M. Starodub, A.V. Rebriiev, Starodu, Ukr. Biokhim. 72 (2000) 88–91.