Co-Immobilization of Cholesterol Esterase, Cholesterol Oxidase and Peroxidase onto Diverse Matrices for Determination of Total Cholesterol

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ABSTRACT

The activity and stability of cholesterol esterase, cholesterol oxidase and peroxidase on co-immobilized onto different matrices such as nylon-6,6 beads and gelatin film coated on cellulose acetate membrane have been investigated. The activity and stability of co-immobilized enzymes by various coupling methods by ascorbic acid or glutaraldehyde for polyamine matrices were used. Commercially available mixture of these three enzymes, as Reagent 1 of kit for cholesterol determination was used. The percentage immobilization varied with the method or matrix. Ascorbic acid coupling of gelatin film was able to retain 67% of the enzymes on immobilization while glutarladehyde coupling to nylon beads 58% retained. The glutaraldehyde coupling of nylon-6,6 showed practically leaching of the enzymes retaining the activity above a period of 30 days, while co-immobilized enzymes were ascorbic acid coupled to nylon-6,6 beads and gelatin film stored were stable over a period of 20 days. The co-immobilized enzymes retained activity more an extensive pH and temperature range and were more stable than the free enzymes at lower and higher pH and temperature values. Co-immobilized enzymes had a higher K_m (36mM) than that of the soluble enzymes (29mM). The co-immobilized enzymes investigated coefficients of variation, linearity, precision, recovery and interference studies.

KEYWORDS: cholesterol oxidase, cholesterol esterase, co-immobilization, ascorbic acid, covalent coupling, glutaraldehyde coupling.

INTRODUCTION

The determination of serum cholesterol is one of the tools in the diagnosis a classification of lipemia. Cholesterol is an unsaturated steroid alcohol and it promotes atheroma development in arteries. It is significant precursor of a large number of steroids which include the bile acids, adrenocortical hormones, vitamin D and cardiac glycosides [1]. Serum cholesterol can be accurately measured enzymatically using cholesterol oxidase and cholesterol esterase. Cholesterol esterase (sterol ester hydrolase; EC 3.1.1.13) a bile salt-dependent lipase, which has a broad specificity towards the various fatty acid residues is used to cleave cholesterol esters to free cholesterol. Subsequently, Cholesterol oxidase (EC 1.1.3.4), transforms the steroid alcohol into cholest-4-ene-3-one and hydrogen peroxide which can be quantitated by a spectrophotometric assay e.g. the oxidative coupling of 4-amino antipyrine and phenol in the presence of peroxidase to form a chromogen [2-4]. Co-immobilization of enzymes is a widely applied technique for catalytic reactions. Advantages of co-immobilized enzymes include reuse, enhanced stability and rapid separation of the catalyst from the reaction mixture. Theoretically, covalent coupling of the enzymes for matrices offers stability. The co-immobilization of enzymes on some insoluble support provides their reuse and thus reduces the cost of procedure. So far only cholesterol esterase and cholesterol oxidase have been immobilized or co-immobilized onto various matrices [5-13]. The present study is a comparative evaluation of the co-immobilization of cholesterol esterase, cholesterol oxidase and peroxidase on various matrices by different coupling methods. The matrices used include (i) nylon-6,6 beads and (ii) gelatin film coated on cellulose acetate membrane and. Ascorbic acid and glutaraldehyde were used for coupling of enzymes on to polyamino matrices of nylon and gelatin film. Kinetic studies was carried out using enzymes co-immobilized on nylon-6,6 beads.

MATERIALS AND METHODS

Chemicals

Cholesterol determination kit was from Beacon diagnostics (P) Ltd., India; nylon beads used for immobilization were gifted by Sriram fibers, gelatin coated on photographic film were obtained locally. The 4-aminophenazone, cholic acid, 25% glutaraldehyde, Triton X-100, Folin Ciocalteu reagent and other chemicals used were from Merck, SRL, CDH and SIGMA in analytical grade purity. Blood samples were collected from Health centre, Anna University. All the solutions were prepared using double-distilled water.

Enzymes

The Reagent 1 of kit for cholesterol determination containing cholesterol esterase, cholesterol oxidase, peroxidase and chromogen and buffer was dissolved in 4 ml 50 mM sodium phosphate buffer, pH 7.0 (4 mg/4 ml) and stored at 4°C.

Preparation of cholesteryl acetate solution

Cholesteryl acetate be use as a substrate for cholesterol esterase. 50 mg of cholesteryl acetate was dissolved in 1.0 ml of Triton X-100 containing 50 mM cholic acid by slowly heating and stirring, until the solution was clear. Sodium phosphate buffer (0.05M, pH 7.0)

was added to get a final concentration of 500 mg/dl. Solutions of different concentration ranging from 50 to 500 mg/dl were prepared similarly and stored at 4 °C.

Preparation of colour reagent

The colour reagent was prepared according to Bias et al. [14] and consisted of 50 mg 4-aminophenazone, 100 mg phenol per 100 ml of 0.4 M sodium phosphate buffer (pH 7.0) and stored at 4 °C.

Combined assay of cholesterol esterase, cholesterol oxidase, peroxidase

The combined assay of enzymes was reaction mixture containing 1.2 ml sodium phosphate buffer (0.05M, pH 7.0), 0.2 ml dissolved enzymes were pre incubated at 37°C for 2 min. The reaction was started by adding 0.1 ml cholesteryl acetate solution (400 mg/dl). 1.0 ml colour reagent was added and the reaction mixture was kept in dark at 37 °C for 10 min to develop the colour. Absorbance was read at 520 nm with the blank solution as reference using a Systronics UV-Visible spectrometer type 118.

Co-immobilization methods

Glutaraldehyde mediated co -immobilization of enzymes on nylon beads

Hydrochloric acid activated nylon beads were immersed in coupling buffer containing glutaraldehyde (1.25% V/V in 50 mM phosphate buffer, pH 7.0) for 40 minutes and washed successively with water and coupling buffer. The glutaraldehyde coupled nylon beads were immersed in the Reagent 1 (0.3 ml) of kit for cholesterol determination containing cholesterol esterase, cholesterol oxidase, peroxidase and chromogen solution and kept overnight at 4°C.

Glutaraldehyde mediated co-immobilization of enzymes on gelatin film

Hydrochloric acid activated gelatin film was immersed in coupling buffer containing glutaraldehyde (1.25% V/V in 50 mM phosphate buffer, pH 7.0) for 40 minutes and washed with water and coupling buffer. The glutaraldehyde coupled gelatin film was immersed in the Reagent 1 (0.3 ml) of kit for cholesterol determination containing enzymes and kept overnight at 4°C.

Ascorbic acid coupling of enzymes on nylon beads

Nylon beads were partially hydrolyzed with hydrochloric acid (2.5 M) at 27º C for about 20 minutes. The beads were washed with water and immersed in ascorbic acid solution (5%) in a coupling buffer of phosphate buffer (50 mM, pH 7) containing 1 mM EDTA for 40 minutes. The beads were collected on a filter and washed with water and buffer. The beads were then immersed in the Reagent 1 of kit for cholesterol determination containing enzymes (0.3ml) co-immobilized nylon beads were washed with water, sodium chloride (1 M) and finally with coupling buffer and kept overnight at 4°C.

Co-immobilization of enzymes onto ascorbic acid coupling of gelatin film

Gelatin film was partially hydrolysed with hydrochloric acid (2.5 M) at room temperature for about 20 minutes. The bead was wash with water and immerse in ascorbic acid solution (5%) in a combination buffer of phosphate buffer (50 mM, pH 7) contain 1 mM

EDTA for 40 minutes. The film was collected on a filter and washed with water and coupling buffer. The film was then immersed in the Reagent 1 of kit for cholesterol determination containing enzymes (0.3ml) extract and kept overnight at 4°C.

Determination of protein bound to different matrices

The protein bound to different matrices was estimated by determining the loss of protein from the solution during enzymes immobilization. For this purpose, the protein content in the native enzymes solution was determined before and after co-immobilization using the method of Lowry *et al*. [15].

Assay of co-immobilized enzymes

Co-immobilized enzymes (1g) were added 1.2 ml 0.05M sodium phosphate buffer, pH 7.0 and 0.1 ml cholesteryl acetate solution. It was pre-incubated at 37°C for 2 min. 1 ml colour reagent was added and incubated for 10 min at 37°C under constant stirring. The colored reaction mixture was withdrawn from the flask using micro pipette transferred it to a cuvette. Absorbance was read at 520 nm with the blank solution as reference using a Systronics UV-Visible spectrometer type 118.

Determination of total cholesterol in serum with co immobilized enzymes

The total cholesterol in serum was determined using co-immobilized enzymes on different matrices in place of native enzymes. 1.0 ml of blood was withdrawn intravenously and centrifuged at 5000 rpm for 5min in a table centrifuge at 27^{o}C. The serum (0.02 ml) was added to 0.98 ml of 50 mM phosphate buffer, pH 7.0, containing 50 mM cholic acid. The assay for total cholesterol in serum was carried out similarly as for co-immobilized enzymes except that the substrate was replaced with pretreated serum. Total cholesterol in serum was extra plotted from a standard curve of cholesterol acetate concentration ranging from 50 to 500 mg/dl against absorbance 520nm.

RESULT AND DISCUSSION

Ascorbic acid mediated immobilization of enzymes on poly amino matrice

The surface activation of amino polymer the free amino group obtained by partial hydrolysis of polyamide (nylon 6,6 beads) and free amino group of gelatin coated on cellulose acetate membrane react with ascorbic acid convert to dehydroascorbic acid products by oxygen utilized autoxidation. The surface activated amino polymer was washed with water and buffer. In the co-immobilization step the activated amino polymer reacts with the enzymes. In this step the β - keto groups of the dehydroascorbic acid derivatives of the amino polymer surface reacts with lysine residues in the enzymes convert to Schiff's base structures (Figure 1). The structures are unstable and convert to oxalic acid diamide bridge between the amino polymer and the enzymes due to an autoxidative fragmentation reaction [16].

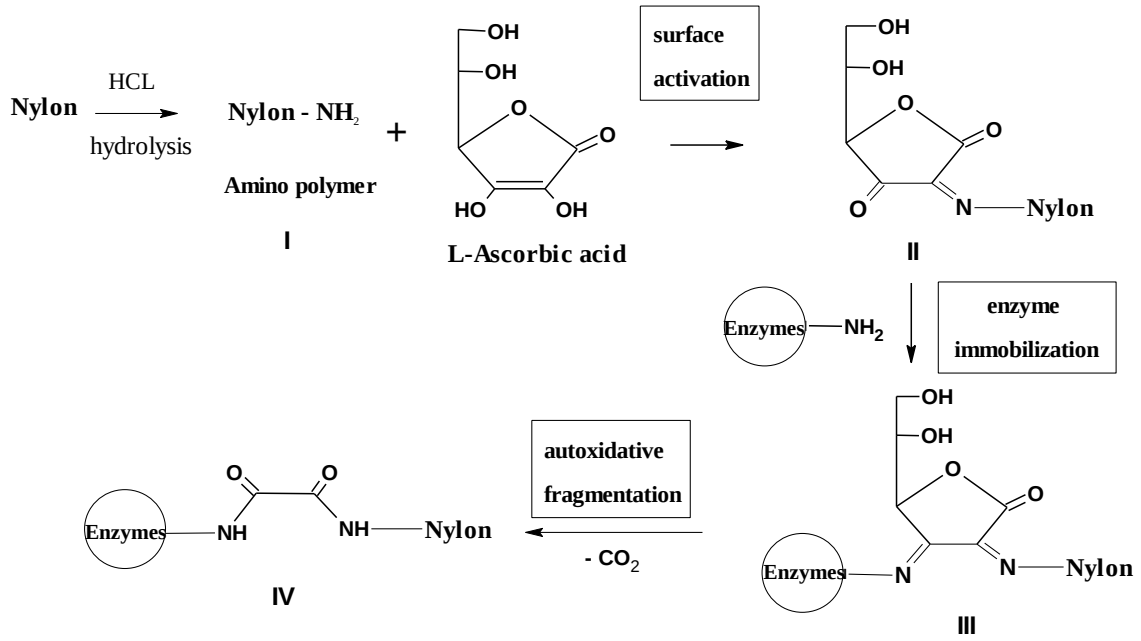

Figure 1. Co immobilization of enzymes on an amino polymer by bifunctional L – ascorbic acid reaction

Immobilization efficiency and storage stability

Commercially available mixture of enzymes, as Reagent 1 of cholesterol determination, has been co-immobilized on ascorbic acid coupling, glutaradehyde coupling on nylon beads, gelatin supported photographic film through glutaraldehyde and ascorbic acid coupling with a conjugation yield of 1.3, 1.2, 1.5 and 1.4 mg protein / gm support (Table 1) which is comparable to that reported for co-immobilization of cholesterol esterase, cholesterol oxidase onto alkylamine glass (2.3 mg protein / gm) (Suman, C.S, 2003). The percentage immobilization varied with the method/ matrix. Ascorbic acid coupling of gelatin film was able to retain 67% of the enzymes on immobilization whereas glutaraldehyde coupling to gelatin film was retained at 65%. (Table 2)

Support	Combined enzymes added to 1 g support (mg)	Co-immobilized enzymes coupled to 1 g support (mg)	Retention of specific activity, %
Nylon bead by ascorbic acid coupling	2.4	1.3	54
Nylon bead by glutaradehyde coupling	2.4	1.2	50
Gelatin film by ascorbic acid coupling	2.4	1.5	62
Gelatin film by ascorbic acid coupling	2.4	1.4	58

Table 1 Protein concentration of combined cholesterol esterase

The long-term stability of co-immobilized matrices was investigated. The matrices were kept for 50 days at 4°C. The enzymes activities were almost the same as the initial activities up to 10 days. After 10 days the enzymes activity at 4°C started to decrease with

time, and almost no activity was observed on day 50 (Table 3). In a different experiment, at 27^{o}C, the co-immobilized enzymes activity was stable up to 10 days and retained almost 50% activity after 30 days, as compared with native enzymes activity. The glutaraldehyde coupling of nylon-6,6 showed practically leaching of the enzymes retaining the activity above a period of 30 days, while co-immobilized enzymes were ascorbic acid coupled to nylon-6,6 beads and gelatin film stored were stable over a period of 20 days.

Support	Co-immobilized enzymes activity, mg/ ml	Un bound enzymes activity, mg / ml	Free enzymes activity, mg/ml	Efficiency of immobilization,%
Nylon bead by ascorbic acid coupling	1.9	1.5	3.5	55
Nylon bead by glutaradehyde coupling	2.0	1.4	3.5	58
Gelatin film by ascorbic acid coupling	2.1	1.4	3.5	59
Gelatin film by ascorbic acid coupling	2.3	1.2	3.5	65

Table 2 Activity and Efficiency of immobilization of co-immobilization onto diverse matrices

Support	1 Day	2 Day	3 Day	4 Day	5 Day	10 Day	15 Day	20 Day	30 Day	40 Day	50 Day
Nylon bead by ascorbic acid coupling	3.21	3.07	3.02	2.77	2.42	2.29	2.03	1.85	1.47	1.05	0.47
Nylon bead by glutaradehyde coupling	3.81	3.67	3.27	3.07	2.92	2.69	2.23	2.01	1.82	1.21	0.81
Gelatin film by ascorbic acid coupling	3.58	3.67	3.29	3.18	3.02	2.87	2.43	2.01	1.62	1.11	0.74
Gelatin film by ascorbic acid coupling	3.68	3.51	3.13	3.05	2.88	2.56	2.29	1.96	1.32	0.96	0.65

Table 3 Time of co-immobilized enzymess activity, mg/ml

Effect of pH on co-immobilized enzymes

An optimum pH range for free enzymes activity was given between pH 6 and 7, while co-immobilized enzymes was present to show activity over a pH range between pH 5 and 8 for most of the matrices. In the case of co-immobilized enzymes on glutaraldehyde coupling of nylon beads and gelatin film maximum activity was found to occur between pH 6 and 8 while ascorbic acid coupling of co-immobilized enzymes on nylon beads and gelatin film exhibited maximum activity over a pH range of 5 to 8 (Figure 2). The activity of the free enzymes was found to decrease by about 50% at pH 5 and 8 compared to its maximum activity at optimum pH of 6-7. In contrast the co-immobilized enzymes showed much greater

activity over the pH range investigated (5-10) the activity decreasing only by 20% at low or higher pH values compared to the maximum activity at the optimum pH. Thus co-immobilization has altered pH optimum in relation to its pH optimum in solution. The behavior of an enzymes molecule may be modified by its immediate micro-environment. It is possible that depending on the surface and residual charges on the solid matrix and the nature of the bound enzymes, the pH value in the immediate vicinity of the enzymes molecule may change the pH optimum of the enzymes [17].

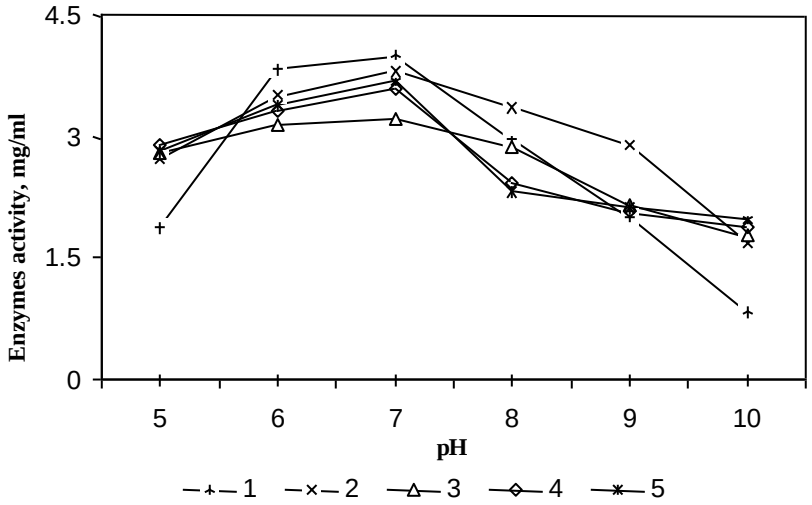

Figure 2. Effect of pH for immobilized free and co-immobilization activity, (mg / ml)

1. Free enzymes 2. Glutaraldehyde coupling on nylon beads

3. Ascorbic acid coupling on nylon beads 4. Glutaraldehyde coupling on gelatin film

5. Ascorbic acid coupling on gelatin film

Effect of temperature on co-immobilized enzymes

The immobilized enzymes were found to be relatively more stable than the free enzymes over a wider range of temperature (Figure 3). The optimum temperature range for free enzymes activity was found to be 30-40 ^{o}C compared to that of the co-immobilized enzymes of 20 to 50 ^{o}C. The free enzymes activity was found to decrease by about 70% at 10^{o}C and at 60^{o}C from the maximum activity at it optimum temperature of 30 ^{o}C. The loss in activity of the co-immobilized enzymes was only by 20% under similar conditions. Enhanced thermal stability has been reported for several covalently bound co-immobilized enzymes [18].

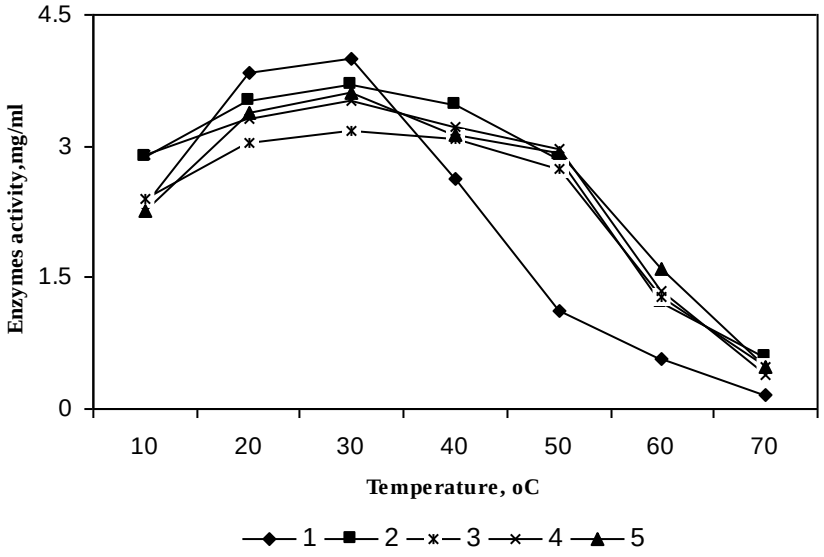

Figure 3. Effect of temperature for free and co-immobilized enzymes activity, mg / ml

1. Free enzymes 2. Glutaraldehyde coupling on nylon beads

3. Ascorbic acid coupling on nylon beads 4. Glutaraldehyde coupling on gelatin film

5. Ascorbic acid coupling on gelatin film

Effect of immobilization on *K*m

Co-immobilized on nylon 6,6 beads showed an apparent K_m value of 30 mM, which is higher than the K_m of the soluble enzymes (20 mM) (Figure 4 and 5). This may be explained on the basis of a concentration gradient of substrate established across the 'Nernst layer', an unstirred layer of solvent surrounding the suspended matrix particles. Consequently, saturation of a co-immobilized enzymes molecule occurs at a higher substrate concentration than normally required for the saturation of the freely soluble enzymes and hence a greater K_m value.

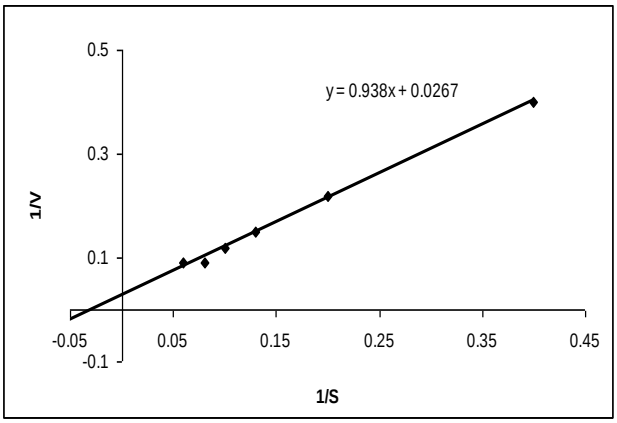

Figure 4. L.B.plot for free enzymes

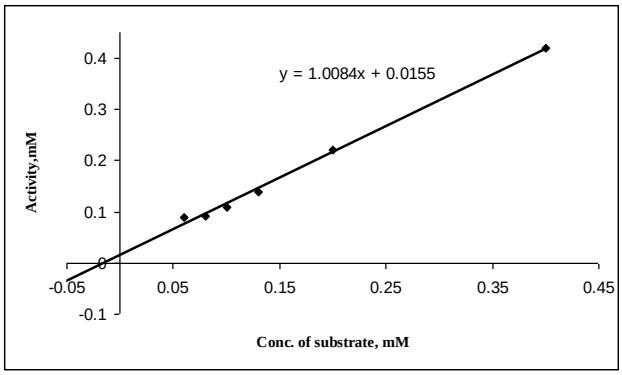

Figure 5. L.B.plot for co-immobilized enzymes

Serum cholesterol value

Blood cholesterol values in healthy male and female individuals of different age groups (10-20, 20-30, 30-40 and 40-50) as measured by our co-immobilized method ranged from 142 to 275 mg/dl with a mean of 189 mg/dl (n = 30), which is in the established normal range 150 to 250 mg/dl. The mean and standard deviations for cholesterol in the total group screened were 189±40 mg / dl.

Linearity and Detection limit

Linearity between cholesterol acetate concentration and absorbance 520nm was obtained between 60 and 370 mg/dl for those using co-immobilized enzymes (Figure 6). The minimum detection limit of the present method is 60 mg/dl, which is comparable to those reported earlier using co-immobilized enzymes 50 mg/dl. [7].

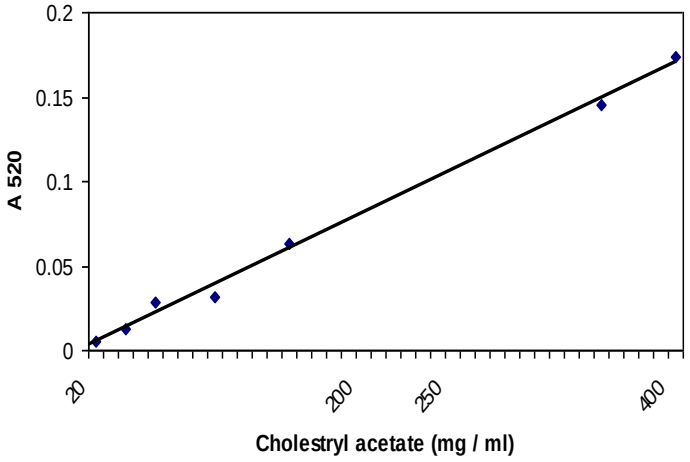

Figure 6. Standard curve for the total cholesterol concentrations on co-immobilized enzymes

Correlation

To estimate the accuracy of the current method, total cholesterol values of 15 serum samples, as determined by the current method utilizing co-immobilized enzymes (y) was compared with marketable enzymes kit (x) utilizing free cholesterol esterase, cholesterol oxidase and peroxidase (Figure 7). Total cholesterol values in serum obtained by other

methods showed good correlation (r = 0.99). This is comparable to the method of Suman C.S, et al., (x) the correlation coefficient 'r' being 0.83. This is comparable to the other method of Abell et al. (x), the correlation coefficient 'r' being 0.99 and with Boehringer Mannheim enzymatic method (x) 'r' being 0.98 [19].

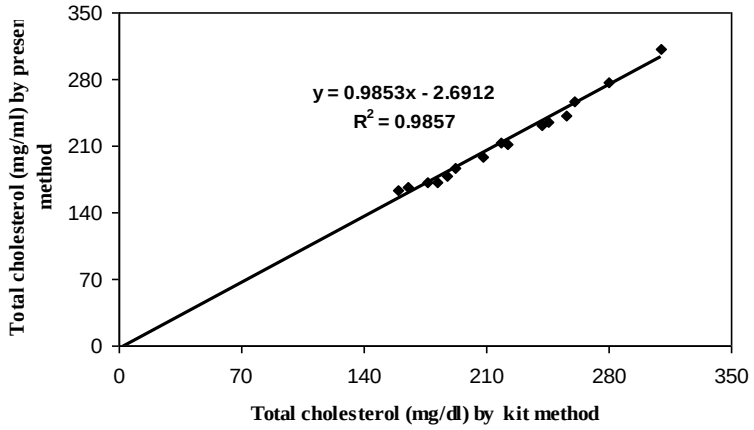

Figure 7. Correlation between blood cholesterol values determined by readymade cholesterol kit and co-immobilized enzymes

Recovery

The proportion of additional cholesterol acetate in serum to mimic hyper cholesterolaemia (50 and 100 mg /dl) by the current method was 94.2 ± 6.1 and 96.2± 4.3 (Mean ± SD) (n = 5) (Table 4), which is comparable with that of GLC method, 98-102 % for additional cholesterol conc. of 50 and 100 mg/ dl [20] and 88.68 – 88.97 % for additional cholesterol conc.100 and 200 mg/ dl (Table 4).

Cholesterol added (mg/dl)	Cholesterol found (mg/dl)	Recovery (%) (Mean ± SD) (n = 5)
None	220	--
50	263	94.2 ± 6.1
100	312	96.2± 4.3

Table 4 Analytical recovery of added cholesterol in serum using co-immobilized cholesterol esterase, cholesterol oxidase and peroxidase

Precision and reproducibility

In order to assess the reproducibility and reliability of the method, the total cholesterol of the same serum sample in one run (within-day variation) and after 1 week of storage (between-day variation) were determined (Table 5). The variation within and between batch CV for total cholesterol determination in serum by the present method were <1.5% and <3.0% respectively which is comparable to those employing nylon mesh immobilized enzymes [21].

Parameter studied	Total cholesterol by co-immobilized enzymes
Within-day (n =5)	
Mean (mg / dl)	184.5
C.V. (%)	1.08
S.D. (mg / dl)	0.02
Between-day (n =5)	
Mean (mg / dl)	226.6
C.V. (%)	2.6
S.D. (mg / dl)	5.9

Table 5 Precision data of total serum cholesterol by co-immobilized cholesterol esterase, cholesterol oxidase and peroxidase

Interference study

To test the possible interference by various metabolites found in serum, the following compounds were added in the reaction mixture at a final concentration 25 µl /2.5 ml of reaction mixture and at their respective physiological concentrations: glucose, citrate, vitamin C, vitamin D, hemoglobin, albumin, sodium bicarbonate, urea, uric acid and creatinine. Of these compounds, none of the metabolites had much effect, except vitamin C, vitamin D and albumin caused 38%, 40% and 30% inhibition of the co-immobilized enzymes system, which is similar with the earlier report [7].

CONCLUSION

The co-immobilized enzymes described in the present study have been shown to retain its activity. The study on the different covalent coupling methods on different matrices indicated that gelatin films and nylon beads to be relatively more efficient. Nylon bead co-immobilized enzymes were found to retain its activity even after 50 days. Co-immobilized enzymes retain its activity over a wider pH and temperature ranges compared to the free enzymes. These methods are applied to the analysis of serum cholesterol in blood. The determination of serum cholesterol in values was compared to the commercially available kit. The data issuing from the various investigations were analyzed using statistical methods and the results were indicated satisfactory performance of the preparation. To sum up, a method has been developed for determination of total cholesterol in serum employing co-immobilized cholesterol esterase, cholesterol oxidase and peroxidase was commercially important.

REFERENCES

1. Deepti Sharma, Jongmin Lee, Junyoung Seo and Heungjoo Shin. (2017) Sensors, 17, 2128.

2. Allain, C.C., Poon, L.S., Chan, C.S.G., Richmond, W., Fu, P.C. (1974) Clin. Chem. 20, 470–475

3. Sundaram, P.V. and Hornby., W. E. (1970) FEBS Lett, 10, 325–329

4. Mattiasson, B., Danielsson, B. and Mosbach, K. (1978) Enzyme thermistorbassay of cholesterol, oxalic acid, glucose and lactose in standard solution and biological samples, in: E.K. Pye, H.H. Weetal (Eds.), Enzyme Engineering, Plenum Press, New York, , pp. 453– 460

5. Simultaneous co-immobilization of three enzymes onto a modified glassy carbon electrode to fabricate a high-performance amperometric biosensor for determination of total cholesterol Khodabakhsh Rashidi , Majid Mahmoudi Int J Biol Macromol (2018) Dec(120) 587-597

6. Rahman, M.M.; Asiri, A.M. One-step electrochemical detection of cholesterol in the presence of suitable K3Fe(CN)6/phosphate buffer mediator by an electrochemical approach. Talanta 2015, 140, 96–101.

7. Soorya, V.C.; Berchmans, S. Flower like Bi structures on Pt surface facilitating effective cholesterol Biosensing. Mater. Sci. Eng. C 2016, 64, 183–189

8. Soylemez, S.; Hacioglu, S.O.; Kesik, M.; Unay, H.; Cirpan, A.; Toppare, L. A novel and effective surface design: Conducting polymer/β-cyclodextrin host-guest system for cholesterol biosensor. Appl. Mater. Interface 2014, 6, 18290–18300.

9. Gholivand, M.B.; Khodadadian, M. Amperometric cholesterol biosensor based on the direct electrochemistry of cholesterol oxidase and catalase on a graphene/ionicliquid-modified glassy carbon electrode. Biosens. Bioelectron. 2014, 53, 472–478.

10. Selvamurugan C, Lavanya A and Sivasankar B. (2007), 'A comparative study on immobilization of urease on different matrices', Journal of Scientific and industrial research, Vol.66, pp. 655-659.

11. Lavanya A, Selvamurugan C and Sivasankar B. (2007), 'Immobilized trypsin mediated production of protein hydrolysates from non-edible protein sources', Journal of Scientific and industrial research, Vol.66, pp. 651-654.

12. Sharma, R.; Sinha, R.K.; Agrawal, V.V. Mediator-free total cholesterol estimation using a bi-enzyme functionalized nanostructured gold electrode. RSC Adv. 2015, 5, 41786–41794.

13. Subramaniam, P.; Sharma, A.; Kaje, K. Association of salivary triglycerides and cholesterol with dental caries in children with type 1 diabetes mellitus. Spec. Care Dent. 2015, 35, 120–122.

14. Bias, R., Potezny, N., Edward, J.B., Rofe, A.M. and Conyers, R.A.J. (1980) Anal. Chem. **52** 508–511

15. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. **193** 265–275

16. Jorg Tiller, Peter Berlin and Dieter Klemm. (1999), Biotechnol Appl Biochem. **30**, 155-162

17. Mosbach, K. (1971) Sci Am, **224,** 26-33

18. Malik, V., Singh, S., Pundir, C.S. (2002) Chin. J. biotechnol. **18**, 155-161

19. Majkic, N. and Berkes, I. (1977) Clin. Chim. Acta **80**, 121–131

20. Blomhoff, J.P. (1973) Clin. Chim. Acta **43,** 257–265

21. Mascini, M., Tomassetti, M. and Lannello, M. (1983) Clin. Chim. Acta 132, 7–15.