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Indian Journal of Clinical Anatomy and Physiology

Journal homepage: www.ipinnovative.com

Original Research Article

Immobilization of bile acid enzymes onto activated amino-ethyl cellulose and polyvinyl alcohol membranes

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ARTICLE INFO

Article history:
Received 22-09-2020
Accepted 25-09-2020
Available online 17-10-2020

Keywords:
3-alpha hydroxysteroid dehydrogenase
Lipoyl dehydrogenase
Bile acid
Amino-ethyl cellulose
Polyvinyl Alcohol

ABSTRACT

Immobilization of bile acid enzymes named, 3-alpha hydroxysteroid dehydrogenase (3-α HSD) and lipoyl dehydrogenase (Diaphorase) enzymes was done on to activated polymeric membranes like amino-ethyl cellulose and Polyvinyl Alcohol. Immobilization of bile acid enzymes was done onto activated amino-ethyl cellulose by covalent coupling by using NaOH and glutaraldehyde. And, Immobilization of bile acid enzymes was done onto activated polyvinyl alcohol by using terephthaladehyde and 2-(m-aminophenyl)-1-3-dioxolane. Percent of enzyme coupled was found 65% and 60% for enzymes coupled onto activated amino-ethyl cellulose and activated polyvinyl alcohol respectively. Observed percent of analytical recovery for bile acid estimation was <98% for activated polymeric membrane bound bile acid enzymes. Improved % of retention activities were observed for activated polymeric membranes bound bile acid enzymes in our present method even after the regular use of more than 4 months (16 weeks) when preserved at 4 dgree C. Hence, our present method of immobilization of bile acid enzymes onto activated polymeric membranes were found to be more cost effective as compared to other reported enzyme based methods.

1. Introduction

Bile acid estimation in serum and bile samples is measured for diagnosis of various hepatobiliary diseases like liver cirrhosis, liver carcinoma, cholelithiasis as well as many adrenocortical diseases and illeal disorders. Bile acid enzyme named, 3-alpha hydroxysteroid dehydrogenase (3-α HSD) and lipoyl dehydrogenase (Diaphorase) has been used for the estimation of bile acid in various biological samples. Hence, various methods have been proposed to immobilized the bile acid enzymes onto various organic and inorganic supports or matrices to reduced the cost of the tests and improving analytical efficiency.[1,2] In last decade, development of various activated polymeric membranes have been chosen for enzymes immobilization for imparting low operational costs, improved preservation or storage ability, increasing enzyme thermal stability.[3] Previously, various enzyme immobilization techniques were proposed for enzyme immobilization involving physical entrapment and chemical interactions like covalent or non-covalent bindings. And, the chosen methods were also found to reported for imparting the low risk of microbial contamination to facilitate more cost effective % of recovery and reusability as well as less non-toxic, inert and biodegradable alternative among various organic and inorganic matrices.[4] Bioluminescence assay was also observed more rapid, highly sensitive, more specific having minimal use of inexpensive reagents to carry out more cost-effective method to estimate bile acid concentration in biological samples to be considered an ideal method.[5] Although many methods have been used for bile acid measurement in biological samples like serum and bile by using high-pressure liquid chromatography (HPLC), gas liquid chromatography (GLC), gas-liquid chromatography–mass spectrometry (GLC-MS) and radioimmunoassay (RIA). The efficacy of bile acids determination for clinical diagnostic purpose were played crucial role in the evaluation of liver function dysfunctions to sketch exact profile of

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https://doi.org/10.18231/j.ijcap.2020.059

the different bile acids to discuss their respective clinical importance in diagnosis of various hepatobiliary diseases, adrenocortical diseases and illeal dysfunctions.[6]

2. Materials and Methods

2.1. Chemicals

3-alpha hydroxysteroid dehydrogenase (3-α HSD) from *Pseudomonas testosteroni* (EC 1.1.50); lipoyl dehydrogenase (Diaphorase) from *Clostridium spp.* (EC 1.8.1.4) (Sigma Chemicals USA); Amino-ethyl cellulose (AMC); Polyvinyl alcohol (PVA); Nitro blue tetrazolium salt (NBT); NAD^+; NADH; Sodium hydroxide (NaOH); Glutaraldehyde; potassium monophosphate (KH_2PO_4); Potassium Biphosphate ($K_2HPO_{4)}$; Hydrochloride (HCl); Terephthaladehyde; 2-(m-aminophenyl)-l, 3-dioxolane (Sisco Research Lab, Mumbai) were used which were of all analytical reagent grade.

2.2. Assay of free 3-alpha hydroxysteroid dehydrogenase (3-α HSD) and lipoyl dehydrogenase (Diaphorase)

Assay of 3-alpha hydroxysteroid dehydrogenase (3-α HSD) was done according to Rani K et al., 2004 and Rani K et al., 2006.[2,3] Assay was based on coupled reactions involving conversion of 3-alpha hydroxy bile acid with NAD^+ into 3-Ketoacid acid and NADH and H^+ in presence of 3-alpha hydroxysteroid dehydrogenase (3-α HSD) followed with the treatment of nitro-blue tetrazolium (NBT) which led to form chromophore (a type of Formazan) and NAD^+ lipoyl dehydrogenase (Diaphorase) measured at wavelength of 540nm. Concentration of bile acid was calculated by using standard curve of taurodeoxycholic acid (micro mol/L) prepared by Rani K et al., 2004 and Rani K et al., 2006.[2,3]

2.3. Preparation of activated amino-ethyl cellulose membrane

Activation of amino-ethyl cellulose was done with 10 ml of sodium hydroxide (NaOH) (in the range of 0.1M to 1.0M) as mediator along with 9-10 ml of 0.08 M phosphate buffer having neutral pH and left for further overnight incubation at 4 degree C to get activated membrane.

2.4. Immobilization of 3-alpha hydroxysteroid dehydrogenase (3-α HSD) and lipoyl dehydrogenase (Diaphorase) onto activated amino-ethyl cellulose membrane

Immobilization of bile acid enzyme named, 3-alpha hydroxysteroid dehydrogenase (3-α HSD) and lipoyl dehydrogenase (Diaphorase) was done onto activated amino-ethyl cellulose membrane by adding 1 to 2 ml of an aqueous solution of 15% of glutaraldehyde was added followed by continuously stirring the solution at room temperature. After stirring, 5ml of 3-alpha hydroxysteroid dehydrogenase (3-α HSD) and lipoyl dehydrogenase (Diaphorase) (1mg/ml) in 1:1 ratio were added and final volume was made up to 8 ml with 0.08 M phosphate buffer (pH 7).[2,3]

2.5. Preparation of activated polyvinyl alcohol membrane

Activation of polyvinyl alcohol (PVA) was done by using 15 ml of 100mg/ml of polyvinyl alcohol with 8 ml of 40mM of terephthaladehyde and 8 ml of 1 N HCl in the ranges. It was further incubated overnight at 4°C to get activated membrane.

2.6. Immobilization of 3-alpha hydroxysteroid dehydrogenase (3-α HSD) and lipoyl dehydrogenase (Diaphorase) onto activated polyvinyl alcohol membrane

Immobilization of bile acid enzymes named, 3-alpha hydroxysteroid dehydrogenase (3-α HSD) and lipoyl dehydrogenase (Diaphorase) was done onto of activated polyvinyl alcohol (PVA) whose activation was done by using 15 ml of 100mg/ml of polyvinyl alcohol with 8 ml of 40mM of terephthaladehyde and 8 ml of 1 N HCl in the ranges. After activation step, 5 ml of 2-(m-aminophenyl)-l,3-dioxolane was added dropwise with continuous stirring at 45 degree C for 1 to 2 hours and let it be left for drying overnight for further activation. After overnight incubation, 5 ml of 3-alpha hydroxysteroid dehydrogenase (3-α HSD) and lipoyl dehydrogenase (Diaphorase) (1mg/ml) in 1:1 ration was added with mild stirring for 10 to 20 hrs preferably at 4 degree C.[2,3]

2.7. Assay of membrane bound (activated amino-ethyl cellulose and polyvinyl alcohol) 3-alpha hydroxysteroid dehydrogenase (3-α HSD) and lipoyl dehydrogenase (Diaphorase)

Assay of membrane bound (activated amino-ethyl cellulose and polyvinyl alcohol) 3-alpha hydroxysteroid dehydrogenase (3-α HSD) and lipoyl dehydrogenase (Diaphorase) was carried out as described for assay of native/ free bile acid enzymes according to Rani K et al., 2004 and Rani K et al., 2006 except free bile acid enzymes were replaced by polymeric membrane bound bile acid enzymes.[2,3]

2.8. Determination of serum bile acid by using membrane bound (activated amino-ethyl cellulose and polyvinyl alcohol) 3-alpha hydroxysteroid dehydrogenase (3-α HSD) and lipoyl dehydrogenase (Diaphorase)

Assay of serum bile acid concentration was carried out by using membrane bound (activated amino-ethyl cellulose and polyvinyl alcohol) 3-alpha hydroxysteroid dehydrogenase (3-α HSD) and lipoyl dehydrogenase (Diaphorase) was carried out by incubating 0.5 ml of test samples with activated polymeric membranes bound bile acid enzymes with 0.5 ml of nitro-blue tetrazolium (NBT) colour reagent at 37°C for 15 minutes (optimum conditions) with continuous stirring as described according to Rani K et al.[2,3] Readings (OD) were taken at 540nm and calculation of measured bile acid in test samples was done by using prepared standard curve of taurodeoxycholic acid as described according to Rani K et al.[2,3]

3. Results and Discussion

Observed optimum pH by using our proposed methods (activated amino-ethyl cellulose membrane bound enzymes & activated polyvinyl alcohol membrane bound enzymes) were 8.5 and 9.2 respectively (Table 1) which were found similar with previous reports.[2,3] Observed optimum temperature by using our proposed methods (activated amino-ethyl cellulose membrane bound enzymes & activated polyvinyl alcohol membrane bound enzymes) were 37 degree C for both methods (Table 1) which were found very much comparable with earlier findings.[2,3] Observed optimum time of incubation by using our proposed methods (activated amino-ethyl cellulose membrane bound enzymes & activated polyvinyl alcohol membrane bound enzymes) were 15 minutes for both methods (Table 1) which were found comparable with previous reported observations.[2,3]

Table 1: Observed optimum kinetic properties of activatedaminoethyl cellulose membrane and polyvinyl alcohol membrane bound bile acid enzymes

S.No.	Kinetic Properties	Activated aminoethyl cellulose membrane bound bile acid enzymes	Activated polyvinyl alcohol membrane bound bile acid enzymes
1.	pH	8.5	9.2
2.	Temperature (degree C)	37	37
3.	Incubation time (minutes)	15	15
4.	Thermal stability (degree C)	45	45

Results of % of analytical recovery of our proposed methods (activated amino-ethyl cellulose membrane bound enzymes & activated polyvinyl alcohol membrane bound enzymes) were approximately <98% which were found to be very much similar to previous findings for extra added bile acid 50 micromol/L and 200 micromol/L in the chosen five test samples by using both of immobilized enzymes (Table 2).[2,3,6,7]

Table 2: Percent of Analytical recovery of added bile acid salt in the known test samples (50micromol/L and 200micromol/L) by using our proposed methods

S. No.	Samples test recovery % (mean±SD) (n=5) *(activated amino-ethyl cellulose membrane bound enzymes & activated polyvinyl alcohol membrane bound enzymes)
50 micromol/L	97.3 ± 3.8 & 98± 3
200 micromol/L	98.5 ± 1.1 & 98± 3.5

Calculated % of enzyme coupled of our proposed methods (activated amino-ethyl cellulose membrane bound enzymes & activated polyvinyl alcohol membrane bound enzymes) were observed 65% and 60% respectively which were also very much comparable with previous reported analysis (Table 2).[2,3,7,8] Observed percent of conjugation yield of immobilization of our proposed methods (activated amino-ethyl cellulose membrane bound enzymes & activated polyvinyl alcohol membrane bound enzymes) were 0.39 and 0.36 respectively which were similar with earlier reports when compared. Percent of retention of immobilized enzymes activities of our proposed methods (activated amino-ethyl cellulose membrane bound enzymes & activated polyvinyl alcohol membrane bound enzymes) were also calculated after performing more than 50 tests (when stored for more than 16 weeks at 4 degree C) which were more than 85% and these findings were found to be very much higher and improved with earlier reports (Table 3) (Figure 1).[2,3,7,8]

Table 3: Various observations of Immobilization of 3-alpha hydroxysteroid dehydrogenase and lipoyl dehydrogenase (diaphorase) onto activated aminoethyl cellulose membrane and polyvinyl alcohol membrane

S. No.	Activated amino-ethyl cellulose membrane bound enzymes	Activated polyvinyl alcohol membrane bound enzymes
Enzymes added (mg/ml)	0.6	0.6
Enzymes coupled (%)	65	60
Conjugation yield (mg/g)	0.39	0.36
Retention of activity (%)	86	88

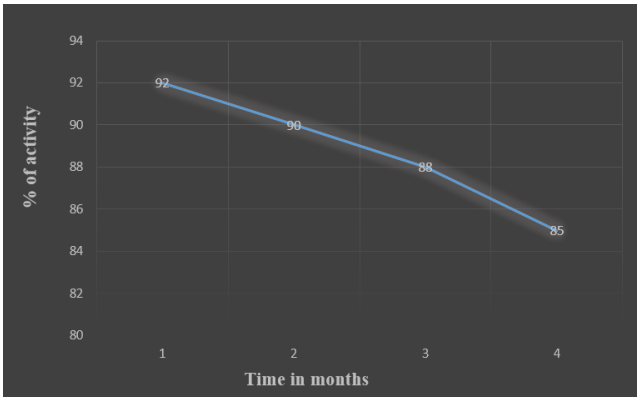

Fig. 1: Observations of Storage stability of activated amino-ethyl cellulose membrane bound enzymes and activated polyvinyl alcohol membrane bound bile acid enzymes

4. Conclusions

Our proposed methods based onto activated amino-ethyl cellulose membrane bound enzymes and activated polyvinyl alcohol membrane bound bile acid enzymes were found to be improved for the percent of immobilization, conjugation yield of coupled bile acid enzymes and % of retention of activity when stored at 4 degree C for more than 4 months (16 consecutive weeks). Hence, these observed results of our proposed methods (activated amino-ethyl cellulose membrane bound enzymes & activated polyvinyl alcohol membrane bound enzymes) were confirmed for their ideal more reliable and cost- effective respective alternatives as compared to previous methods for carrying out more efficient serum bile acid analysis. And, efficacy of bile acids determination in various biological samples might be improved by using our proposed methods (activated amino-ethyl cellulose membrane bound enzymes & activated polyvinyl alcohol membrane bound enzymes) for clinical diagnosis of liver function dysfunctions, hepatobiliary diseases, adrenocortical diseases and illeal dysfunctions as cost effective alternative.[9] As well as these polymeric membranes bound bile acid enzymes methods can also be considered in future for preparation of bile acid biosensor to carry out more reliable and sensitive analytic method for assay of bile acid in various biological samples.

5. Source of Funding

None.

6. Conflict of Interest

None.

Acknowledgment

I would like also to express my cordially appreciation to Amity University Uttar Pradesh, Noida (INDIA).

References

1. Rani K. Review: Bile Acid Analysis. *Int J Pharm Biomed Sci*. 2012;3(2):28–34.
2. Rani K, Garg P, Pundir C. Discrete analysis of bile acid with immobilized 3 α-hydroxysteroid dehydrogenase and diaphorase onto alkylamine glass beads. *Indian J Biochem Biophysics*. 2006;43:98–104.
3. Rani K, Garg P, Pundir CS. Measurement of bile acid in serum and bile with arylamine-glass-bound 3α-hydroxysteroid dehydrogenase and diaphorase. *Anal Biochem*. 2004;332(1):32–7.
4. Trauner M, Claudel T, Fickert P, Moustafa T, Wagner M. Bile Acids as Regulators of Hepatic Lipid and Glucose Metabolism. *Dig Dis*. 2010;28(1):220–4.
5. Roda A, Girotti S, Ghini S, Grigolo B, Carrea G, Bovara R. Continuous-flow determination of primary bile acids, by bioluminescence, with use of nylon-immobilized bacterial enzymes. *Clin Chem*. 1984;30(2):206–10.
6. Street JM, Trafford DJ, Makin HL. The quantitative estimation of bile acids and their conjugates in human biological fluids. *J Lipid Res*. 1983;24(5):491–511.
7. Houten SM, Watanabe M, Auwerx J. Endocrine functions of bile acids. *EMBO J*. 2006;25(7):1419–25.
8. Bartling B, Li L, Liu CC. Determination of total bile acid levels using a thick-film screen-printed Ir/C sensor for the detection of liver disease. *Anal*. 2009;134:973.
9. Zhao RH, Li BY, Chen N, Zhang YK, Wang ZY, Lu PC. High performance liquid chromatographic determination of individual bile acids in serum for automatic diagnosis of various liver and biliary diseases. *Biomed Chromatography*. 1993;7(3):139–42.

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Cite this article: Rani K. Immobilization of bile acid enzymes onto activated amino-ethyl cellulose and polyvinyl alcohol membranes. *Indian J Clin Anat Physiol* 2020;7(3):292-295.