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Determination of Total Cholesterol in Serum by Cholesterol Oxidase and Cholesterol Esterase Co-immobilized on to Cellulose Acetate Membrane

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Abstract: Optimization of the co-immobilized cholesterol esterase and cholesterol oxidase preparations on to cellulose acetate membrane by absorption was performed. A maximum absorption of 75% for cholesterol oxidase and 63% for cholesterol esterase was obtained under the conditions consisting of agitation rate of 70 oscillations per min for 18 h using 0.06 U cm^{-2} of cholesterol esterase and 0.08 U cm^{-2} of cholesterol oxidase at 37°C and pH 7.4. The enzyme activities and stabilities were improved when treated with 1.5% of glutaraldehyde. A significant and satisfactory stability of 8 repeated uses of the co-immobilized preparations was obtained with a $t_{½}$ of 15 consecutives uses. When compared to the commercially available Sera-Pak® (*Bayer corporation*) and CHOD-PAP (*Chema diagnostica*), the co-immobilized enzymes preparation showed a significant correlation with an r value of 0.95 and 0.99, respectively.

Key words: Enzyme co-immobilization, cholesterol esterase , cholesterol oxidase, cellulose acetate membrane, cholesterol determination

Introduction

It has been well known that immobilization of enzymes has been considered as one of the advancements in enzyme technology. To date there are voluminous information on enzyme immobilization involving new techniques and support materials. However, the objectives of enzyme immobilization remain unchanged that include to improve its stability, activity, reusability and to prevent enzyme deactivation due to environmental perturbation (Chibata, 1978; Klibanov, 1979). The use of immobilized enzymes for analysis was also reported, however most of the enzyme preparations exhibited poor reusability and stability (Tatsuma, 1991; Crumbliss *et al*., 1994). High stability and reusability are among the vital pre-requisite for immobilized enzyme preparations which are used for analytical applications or as biosensors. This short research note describes the co-immobilization of several key enzymes used in the determination of total serum cholesterol. The enzymes involved include cholesterol esterase and cholesterol oxidase which were co-immobilized by the absorption method on to cellulose acetate membrane coated on glass beads. The system was proposed for the development of a simple, cheap and rapid analytical kit for the determination of total cholesterol in serum. The principle for the determination of cholesterol in serum was based on the reaction catalysed by cholesterol esterase and cholesterol oxidase. Cholesterol from cholesterol ester was obtained from the reaction catalysed by cholesterol esterase. The cholesterol will then, be converted to choles-4-en-3-one and hydrogen peroxide by cholesterol oxidase. Hydrogen peroxidase will form a chelate with xylenol orange and quadrivalent titanium dioxide complex and the concentration of the chelated complex can be determined by measuring the intensity of the coloured complex at the wavelength of 550 nm. Based on this principle, co-immobilization of the key enzymes mainly cholesterol oxidase and cholesterol esterase were performed and the performance of the preparations for the determination of total cholesterol in serum was evaluated.

Materials and Methods

Pure lyophilized enzyme preparations of cholesterol oxidase (16.4 U mg^{-1}), cholesterol esterase (20.5 U mg^{-1}) and horseradish peroxidase (220 U mg^{-1}) were generous gifts of Amano Pharmaceutical Co. Ltd., Japan. Human blood samples were obtained from the blood bank of Penang General Hospital, Georgetown, Penang, which was stored at 4°C before used. Blood serum was prepared by centrifugation at 3000 rpm for 5 min at 4°C and the serum was used as the sample for cholesterol determination.

The cross linked absorbed enzymes system on to cellulose acetate membrane was prepared using a mixture of 24 g of cylcohexanone, 24 g acetone and 1 g of cellulose acetate which was mixed thoroughly at room

temperature until a homogenous solution was obtained. Glass beads of 5 mm diameter were added to the mixture and allowed for further mixing. The organic solvents were allowed to evaporate and a thin film or membrane was formed on the surface of the glass beads. The cellulose acetate membrane coated glass beads were agitated at 50 oscillations min^{-1} with either cholesterol oxidase (0.40 U ml^{-1}), cholesterol esterase (0.40 U ml^{-1}) or horseradish peroxidase (110 U ml^{-1}) solutions or a mixture of these enzymes in a water bath at 30°C for 45 h, followed by further agitation for 2 h in the presence of 1% (v v^{-1}) glutaraldehyde. The enzyme activities of the co-immobilized enzymes preparation were determined. The activity of cholesterol oxidase and cholesterol esterase was determined by the modified method of Allain *et al.* (1974). The activity of horseradish peroxidase was determined according to the procedure outlined by Amano Pharmaceutical Co., (Japan). The beads are rinsed with phosphate buffer (pH 7.4) and stored at 4°C in the same buffer prior to use.

The reaction mixture for the determination of cholesterol oxidase activity consists of 3.0 ml 0.1 M phosphate buffer containing 0.5 ml Triton-X-100 L^{-1} buffer (pH 7.0) and free or cholesterol oxidase membrane coated glass beads of known surface area. About 50 μl of the cholesterol (200 mg dL^{-1}) was added and the absorbance of the product, choles-4-en-3-one was monitored at 240 nm at 37°C for a period of 1 min. One unit of cholesterol oxidase is the amount of enzyme which catalyses the formation of 1 μ mol of choles-4-en-3-one under the conditions examined. The molar adsorptivity of choles-4-en-3-one, $\epsilon = 12.2 \times 10^3$ liter $mol^{-1}cm^{-1}$ was used to calculate the activity of cholesterol oxidase. The cholesterol esterase activity was determined using the reaction mixture consisting of 50 μl Serachol (3.0 mmol sodium cholate, 0.8 mmol 4-aminoantipyrine, 14 mmol phenol, 67000 U horseradish peroxidase, 120 U cholesterol oxidase and 0.17 mmol Carbowax-6000) in 3.0 ml of 0.1 M phosphate buffer of pH 6.7. The reaction mixture was allowed at 37°C for 5 min with the addition of free or cholesterol esterase membrane coated glass beads of known surface area. The changes in the absorption was determined at the wavelength of 500 nm at 37°C. One unit of cholesterol esterase is the amount of enzyme which causes the hydrolysis of 1 μmol of cholesterol ester min^{-1} at 37°C. The calculation was based on the adsorptivity of the indicator used which is $\epsilon = 5.33$ Lm mol^{-1} cm^{-1}.

Total serum cholesterol using the co-immobilized cholesterol esterase and cholesterol oxidase (or hydrogen peroxidase) system was determined based on the modified method described by Richmond (1972) using the reaction mixture consisting of 100 μl plasma sample or blank, 500 μl of neutralizing reagent, 500 μl of oxidizing reagent and 1000 μl of the indicator, xylenol orange-titanium dioxide complex. The reaction was allowed for 10 min at 37°C after which the absorbance at 550 nm was measured. The neutralizing reagent was prepared by adding 16.6 ml of 1.0 mol l^{-1} hydrochloric acid to the final volume of 100 ml using Tris(hydroxymethyl) amino methane phosphate buffer (pH 7.6). The oxidizing reagent was prepared by adding 14 mg of mercuric chloride and 1.0 g sodium azide to 100 ml of distilled water.

A standard curve of different concentration of cholesterol (Ajax) between 0.5 - 7.0 mg ml^{-1} was obtained which was allowed to react with cholesterol oxidase of concentration 0.25 U ml^{-1}. The indicator , xylenol orange-titanium dioxide complex of volume 1000 μl was added and allowed for 10 min at 37°C. Absorbance at 550 nm was determined and a standard curve between the concentration of cholesterol against the absorbance was obtained.

The concentration of cholesterol was also determined using the commercially available enzymatic calorimetric methods of Sera Pak®, (Bayer Corporation, USA) and the CHOD-PAP (Chema Diagnostica, Italy) for comparison with the co-immobilized enzymes preparation.

Results and Discussion

The effect of multiple enzymes preparation for the determination of cholesterol was determined (Fig. 1). The co-immobilization of cholesterol oxidase and cholesterol esterase gave the highest cholesterol concentration. Relatively low concentration of the total cholesterol was detected using the co-immobilized enzymes system in the presence of horseradish peroxidase. The co-existences of the 3 enzymes during immobilization resulted in poor absorption on to the membrane, which may be the reason for the low total cholesterol analysed. Thus, hydrogen peroxidase was eliminated in the assay system and the amount of cholesterol present will have to be based on the detection of hydrogen peroxide. The amount of hydrogen peroxide present was detected using an optimized indicator or dye system consisting of xylenol orange (0.76 g l^{-1} in 2% Triton X-100) and titanium dioxide (0.8 g titanium dioxide, 5 g ammonium sulphate in 100 ml concentrated sulphuric acid). The dye complex which was prepared by adding xylenol orange and titanium dioxide at the ratio of 1:1 was able to form chelate with hydrogen peroxide. The intensity of the colour of the chelated hydrogen peroxide-xylenol orange-titanium dioxide complex is proportional to the amount of hydrogen peroxide or cholesterol present. Malik and Pundir (2002) also reported the co-immobilization of cholesterol oxidase and cholesterol esterase on to arylamine glass for the determination of total cholesterol in serum. However,

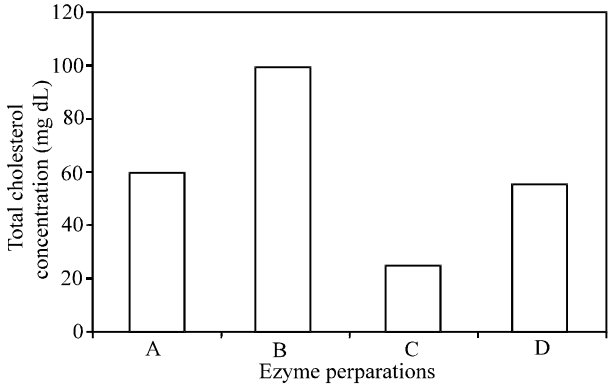

Fig. 1: Effect of co-immobilization of enzymes on to cellulose acetate membrane
A. cholesterol oxidase, B. cholesterol oxidase and cholesterol esterase, C. cholesterol oxidase and horseradish peroxidase, D. cholesterol oxidase, cholesterol esterase and horseradish peroxidase.
The concentration of cholesterol oxidase and cholesterol esterase used was 0.4 U ml^{-1}, respectively while the concentration of horseradish peroxidase was 110 U ml^{-1}

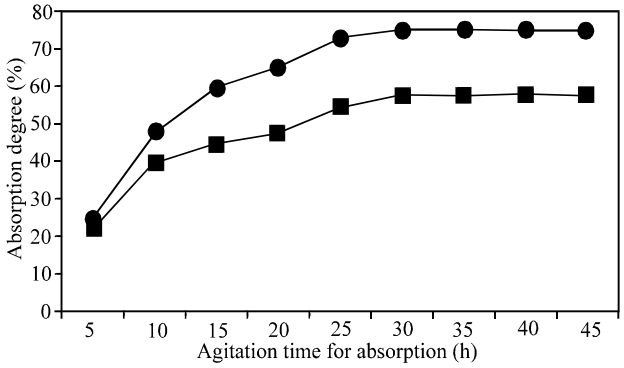

Fig. 2: Effect of agitation time on the co-immobilization of cholesterol oxidase and cholesterol esterase on to cellulose acetate membrane
Immobilization of cholesterol oxidase and cholesterol esterase was carried out at 30°C with an agitation rate of 70 oscillations min^{-1}. Absorption degree was based on the ratio of the activities of the enzymes to the initial enzyme activities used initially for the co-immobilization.
(■) cholesterol esterase (●) cholesterol oxidase

horseradish peroxidase was also employed to determine the amount of hydrogen peroxide using amino phenazone and phenol as the dye system.

Five membrane coated glass beads were agitated with cholesterol esterase and cholesterol oxidase enzyme solutions at 30°C for 45 h at the rate of 50 oscillations

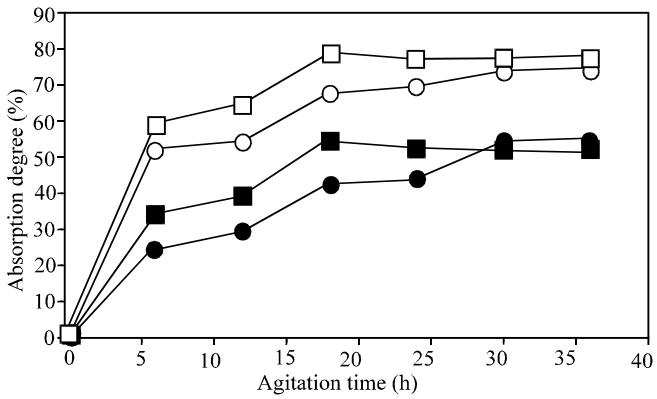

Fig. 3: Effect of temperature on the co-immobilization of cholesterol oxidase and cholesterol esterase on to cellulose acetate membrane
(■) cholesterol esterase (●) cholesterol oxidase at 30°C and (□) cholesterol esterase and (O) cholesterol oxidase at 37°C.

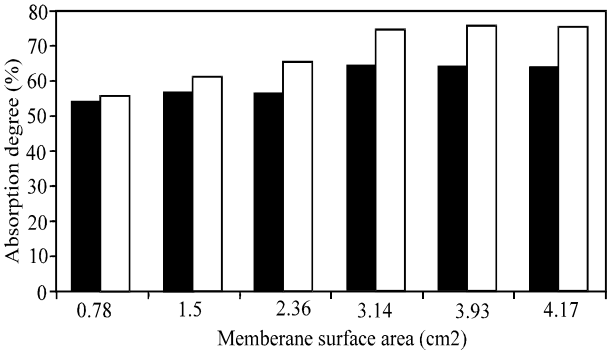

Fig. 4: Effect of membrane surface area on the co-immobilization of cholesterol oxidase and cholesterol esterase on to cellulose acetate membrane
(■) cholesterol esterase (□) cholesterol oxidase
Absorption was carried out 30°C.

min^{-1}. A maximum absorption degree of cholesterol oxidase of about 55% was observed after 30 h. However, when the agitation rate was increased to 60 oscillations min^{-1}, an absorption of about 60% was obtained. On the other hand, when the agitation rate was further increase to 70 oscillations min^{-1}, the absorption was further increased to 75% for cholesterol oxidase and 58% for cholesterol esterase which was achieved after 30 h (Fig. 2). Co-immobilization was also carried out at 37°C at 70 oscillations min^{-1}, and it was observed that the time taken to achieve maximum absorption of 75% for cholesterol oxidase and 58% for cholesterol esterase was significantly reduced from 30 to 18 h (Fig. 3). The absorption of the enzymes on to the membrane which is

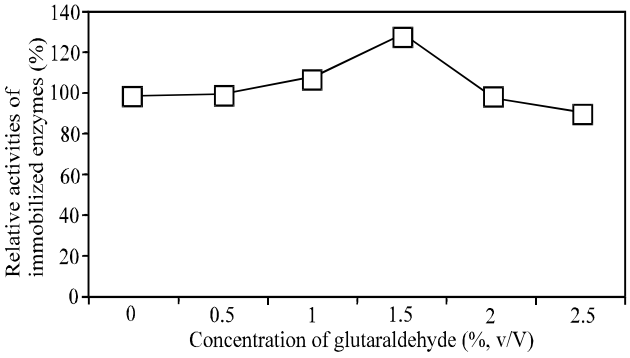

Fig. 5: Effect of glutaraldehyde concentration on the activities of the co-immobilized enzymes preparation
Glutaraldehyde at various concentration was added during the co-immobilization of enzymes on to the cellulose acetate membrane on the glass beads.

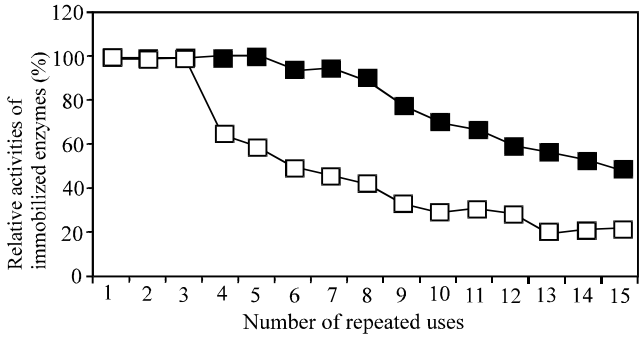

Fig. 6: Reusability of the co-immobilized enzymes preparation for the total cholesterol determination
(□) without glutaraldehyde treatment and (■) with glutaraldehyde treatment

governed by temperature and agitation rate suggested that the mechanism of absorption depends on the active collision of enzymes molecules on to the membrane surfaces. The pH of the buffer used in the preparation of the enzymes also affected the absorption of the enzymes. For cholesterol oxidase and cholesterol esterase, the optimum pH for optimum activities were found to be 7.4 and 7.6, respectively. However, the buffer pH of co-immobilized enzymes system of 7.4 was able to maintain the activities of the enzymes which were absorbed on to the membrane and therefore it was used in the subsequent experiments.

The co-immobilization of the enzymes on to the membrane depends on the surface area of the membrane available for absorption. The experiment was carried out by increasing the number of beads or the total surface area for absorption. It was observed that the use of 5 beads with an equivalent surface area of 3.93 cm^2 resulted in the absorption degree of about 75% for cholesterol oxidase and 63% for cholesterol esterase (Fig. 4). The maximum cholesterol concentration was obtained using the co-immobilized preparations of cholesterol esterase concentration of about 0.06 U cm^{-2} of the membrane and the cholesterol oxidase concentration of 0.08 U cm^{-2}.

The rigidity and the porosity of the membrane are related to the amount of cellulose acetate used in the immobilization. The effect of the amount of cellulose acetate in the range of 0.8 – 1.4 g was examined based on the enzymes activities. It was found that the optimum amount was 1.0 g (in 24 g cyclohexanone and 24 g acetone), of which further amount of cellulose acetate will lead to the drop in the enzyme activities (data not shown). Glutaraldehyde, a bifunctional reagent, is used to form cross linkages between enzyme molecules which were absorbed on to the surface of the cellulose acetate membrane. The stability and activity of immobilized lipases on several support materials have shown to be highly improved with the treatment of glutaraldehyde (Ibrahim *et al.*, 1988; Lawson, 1990). The activities of the absorbed enzymes was found to improve by about 30% using the glutaraldehyde concentration of 1.5% when compared to without glutaraldehyde treatment (Fig. 5). With the glutaraldehyde concentration of 2.0% or more, the enzyme activities of the enzymes remain unaffected. The results suggested that the excessive cross linkages will result alteration in the rigidity and porosity of the membrane structures which affected the accessibility of substrates for catalytic activities of the absorbed enzymes.

The co-immobilized enzymes system of cholesterol esterase and cholesterol oxidase were examined on the reusability of the preparation for cholesterol determination using the same batch of serum sample. After each reaction, the membrane coated glass beads were rinsed thoroughly with phosphate buffer solution (pH 7.4) and was used for the subsequent reactions. The preparation without glutaraldehyde treatment showed 100% stability after only 3 consecutive uses which dropped to about 60% after the 5th usage. On the other hand, the glutaraldehyde treated preparation was found to show 100% stability after 5 consecutive uses, which dropped to about 90% activity after the 8th usage (Fig. 6). The enzyme preparation is considered significantly stable with the half-life ($t_{½}$) of 15 consecutive uses.

Comparison between the co-immobilized enzymes preparation and the commercial kit was done statistically based on the linear regression correlation. The co-immobilized enzymes system consisting of cellulose acetate coated membrane on glass beads of area 3.93 cm^2

(0.06 U cm^{-2} of cholesterol esterase and 0.08 U cm^{-2} of cholesterol oxidase) was used to determine the total serum cholesterol and comparison was made using the commercially available analytical kit Sera-Pak® and CHOD-PAP. The r value for the correlation based on the linear regression between the use of co-immobilized enzymes preparation and Sera-Pak® and CHOD-PAP was 0.95 and 0.99, respectively which indicated satisfactory performance of the preparation. The characteristics of the co-immobilized enzyme systems in terms of its repeated usage is commendable. Some interesting features of the assay system include the short assay time, its simplicity and does not require the use of hydrogen peroxidase enzyme, may be regarded as one of the potential alternative methods for serum total cholesterol determination.

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