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Immobilization of Phospholipase A₂ on Porous Glass and Its Application for Lowering Serum Cholesterol Concentration

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ABSTRACT

Phospholipase A₂ (PLA₂; EC 3.1.1.4) is a lipolytic enzyme that hydrolysis the ester bond in *sn*-2 position of phospholipids. In this work, the PLA₂ from hog pancreas was covalently coupled to porous glass. The properties of free and immobilized enzyme were also investigated and compared. The optimum pH and temperature were found as 8.5 and 50°C, respectively for both free and immobilized enzyme. The immobilized enzyme had good properties that potential for medical application is considerable. Its use in lowering plasma cholesterol concentrations in blood samples was also demonstrated.

Key Words: Phospholipase A₂; Porous glass; Immobilization; Cholesterol.

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INTRODUCTION

The phospholipase A₂ (PLA₂) superfamily consists of a broad range of enzymes defined by their ability to catalyze specifically the hydrolysis of the center (*sn*-2) ester bond of substrate phospholipids (Arni and Ward, 1996; Chen and Chen, 1998; Madoery et al., 1995; Six and Dennis, 2000). It is a lipolytic enzyme that hydrolysis phosphoglycerids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) at the acyl ester bond at the *sn*-2 position into the corresponding lysophospholipid and fatty acid (Kaiser, 1999; Simonsson and Ahren, 2000; Six and Dennis, 2000).

The hydrolysis products of this reaction, free fatty acid and lysophospholipids have many important downstream roles and are derived from the activity of a diverse and growing superfamily of PLA₂ enzymes. The lysophospholipid product is important in cell signalling, phospholipid remodelling and membrane perturbation. The fatty acids can be important as stores of energy, but more importantly arachidonic acid has also function as a second messenger and precursor of eicosanoids, which are potent mediators of inflammation and signal transduction (Balsinde et al., 1999; Capper and Marshall, 2001; Six and Dennis, 2000). Different forms of the enzyme exist, which are classified into a *secretory* (sPLA₂) and a *cytosolic* (cPLA₂) category. The secretory and cytosolic PLA₂s are further subdivided into a number of subtypes depending on structure, mass, substrate specificity and Ca⁺² dependency (Simonsson and Ahren, 2000; Six and Dennis, 2000). The secretory forms of PLA₂ are enzymes of low molecular weight (14 kDa), which require calcium concentrations of milimolar levels for activity and preferentially hydrolyse arachidonic acid containing phosphatidylethanolamine. This group includes several types of sPLA₂ such as those secreted in venoms, by the pancreas and by other mammalian cells. In contrast, the cytosolic forms of PLA₂ are exclusively present inside the cell, are of high molecular weight, need only micromolar or submicromolar Ca⁺² levels for their activation and show a preference for phosphatidylcholine with arachidonic acid in the *sn*-2 position (Kaiser, 1999; Simonsson and Ahren, 2000; Yedgar et al., 2000).

Through its enzymatic activity, or by the generation of arachidonic acid and arachidonic acid metabolites, PLA₂ is known to exert biological effects in several different cell systems: for example, as a digestive enzyme in intestinal juices, as protecting basal plasma membrane homeostasis in mast cell, as constituting the rate limiting event in biosynthesis of pro-inflammatory lipid mediators, and as participating in the enzyme cascades generating transduction signals in, for instance, macrophages, vascular smooth muscle cells, mast cells, and keratinocytes (Dennis, 1994; Simonsson and Ahren, 2000).

The PLA₂s are widely distributed in nature and are present in most animal and human tissue and abundant in mammalian pancreas and in the venom of several snakes and bees (Arni and Ward, 1996; Chen and Chen, 1998). The hydrolytic reaction promoted by PLA₂ has importance in biotechnology since the lysophospholipid properties. The use of lysolipids as food additives is well known. It prevents unwanted colorization of food or contamination with microbial spore and plays a role in taste substances (Kim et al., 2001; Madoery et al., 1995). The new applications of these compounds were also reported in the pharmaceutical industry and in biomedicine (Madoery and Fidelio, 2001; Mukherjee, 1990; Tai and Cooper, 1990).

Moreover, biomedical reactors with immobilized PLA₂ and their application has been only attempted in the treatment of hypercholesterolemia to lower serum cholesterol and low-density lipoproteins in plasma (Chen and Chen, 1998; Labeque et al., 1993; Shefer et al., 1995a,b; Shen and Cho, 1995). Low-density lipoprotein (LDL) particle is the major cholesterol carrier in the plasma. Enzymatic modification of LDL particle with PLA₂, by enzymatic hydrolysis of phospholipid on the particle surface, does not significantly change the basic structural features of the particle, including solubility. Several studies have established that the higher the total plasma cholesterol levels, the greater risk for coronary heart disease. Drug therapy, diet control and direct removal of LDL from the blood can reduce plasma cholesterol level. Drug therapy may have side effects to limit its use. On the other hand, the success of diet control depends heavily on continued and sometimes unpleasant cooperation from patients. However, a promising and interesting route for treating high total plasma cholesterol and LDL-cholesterol levels is to use an extracorporeal enzyme bioreactor which contains immobilized PLA₂ to modify plasma LDL so that the modified LDL may be removed by an individual's own metabolic processes (Chen and Chen, 1998).

In this study, we have immobilized pancreatic phospholipase A₂ on activated porous glass with a high fixation level. The macroporous structure of the support provides good diffusivity of LDL particles. Some properties of the immobilized enzyme were investigated and its use for lowering cholesterol concentration of serum was also demonstrated and compared with the free enzyme.

MATERIALS AND METHODS

Materials

Phospholipase A₂ (PLA₂) from hog pancreas, 3-*sn*-phosphatidylcholine (lecithin) were obtained from Fluka Chemie AG (Switzerland).



Glutaraldehyde and 3-aminopropyltriethoxysilane (APTS) were obtained from Sigma Chem. Co. (St. Louis, USA). Porous glass beads were purchased from Schott Glasswerke (Mainz, Germany). The cholesterol kit (cholesterol liquicolor) was purchased from Human Gessellschaft für Biochemica und Diagnostica GmbH (Germany). Other reagents were of analytical grades and purchased from Sigma Chem. Co. (St. Louis, USA) or E. Merck (Darmstad, Germany).

Methods

Immobilization of PLA₂ on Porous Glass Beads

Porous glass beads were first activated by APTS. The silanization procedure was performed as described by Weethall (1993). The ϵ -amino groups of lysine on enzyme molecules were attached to the aminopropyl glass beads via glutaraldehyde (2.5%) (Figure 1).

The beads (1 g) were first washed several times with distilled water and then suspended in 20 ml of 10% APTS solution. The pH of the medium was adjusted between pH 3.0–4.0 with concentrated HCl and

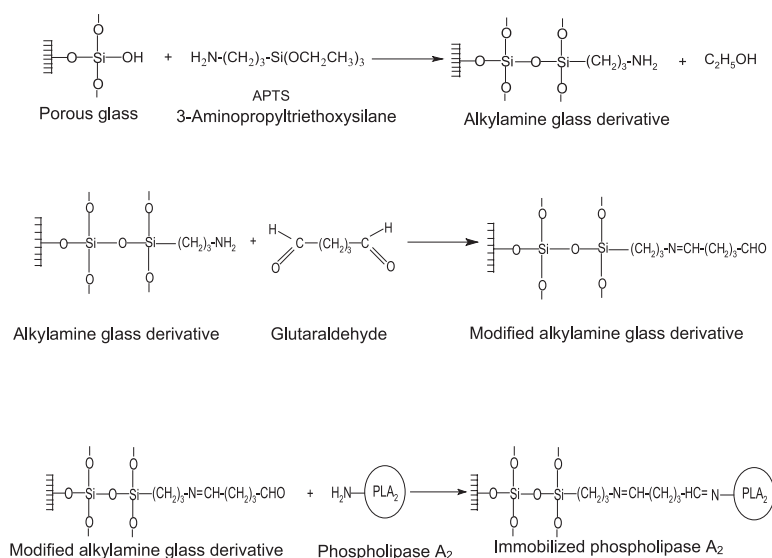


Figure 1. Immobilization of PLA₂ on porous glass.

then was agitated gently (150 rpm) at 75°C for 5 hours. The APTS activated glass beads were washed with distilled water for three times. The aminopropyl-glass derivative was suspended in 25 ml of 2.5% glutaraldehyde solution (prepared in Tris-HCl buffer, 0.1 M, pH 8.0) and was agitated at 200 rpm at room temperature for one hour. The beads were separated and washed with the same buffer. They were added to 10 ml of PLA₂, which prepared in Tris-HCl buffer with gentle shaking and incubated at 4°C overnight. The immobilized enzyme conjugate was separated by filtration and then in order to remove unbound proteins it was washed with the same buffer. The filtrate and washing solutions were collected and used for protein determination.

Activity Measurement of PLA₂

The enzymatic assay used 3-*sn*-phosphatidylcholine (lecithin) as substrate. Lecithin emulsion was prepared as follows: 54 mg of sodiumdeoxycholate (2.6 mM) was dissolved in 50 ml of distilled water and then 1.25 g of lecithin (2.5%) was added. After stirring for 30 minutes at 4°C, the mixture was sonicated for 1 minute. The substrate emulsion was prepared daily. The enzymatic reaction was carried out in a vessel surrounded by a heating jacket through which water at 37°C was circulated. The mixture consists of 9.5 ml of 0.1 M NaCl, 12.5 ml of 0.01 M CaCl₂ and 3 ml of lecithin emulsion. The mixture was stirred magnetically to ensure uniform temperature and material distribution in the reaction vessel and then 250 µl of free enzyme or 100 mg of immobilized enzyme was added. The free fatty acid that is produced in the enzymatic reaction was titrated 0.01 M NaOH using an automatic titration assembly (718 Stat Titrino Methrom, Ltd., Switzerland). Each mole of the substrate was hydrolyzed by the enzyme to yield one mol of fatty acids that can be titrated against one equivalent NaOH. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 µmol of substrate per minute (Chen and Chen, 1998; Kim et al., 2001).

Protein Determination

Protein concentrations were determined by the method of Lowry et al. (1951) by using bovine serum albumin as standard. The amount of bound protein was calculated from the difference between the amount of protein introduced into the reaction mixture and the amount of protein in the filtrate and washing solutions after immobilization.



Cholesterol Measurement

Cholesterol concentrations were measured by using standard cholesterol kit; Cholesterol liquicolor. The cholesterol amounts were determined by using the formulas given below:

$$C = 5.17 \times \frac{\Delta A_{\text{sample}}}{\Delta A_{\text{standart}}} [\text{mmol/l}]$$

$$C = 200 \times \frac{\Delta A_{\text{sample}}}{\Delta A_{\text{standart}}} [\text{mg/dl}]$$

Effect of pH and Temperature on Activity

The effect of pH and temperature on activity of free and immobilized PLA₂ were assayed in 50 mM phosphate buffer (pH 7.0–9.0 and temperature 30–70°C) by using the standard activity assay procedure mentioned above.

Operational Stability

The reaction of lecithin hydrolysis by the porous glass-immobilized PLA₂ was carried out for 6 hours at 37°C in a continuous stirred tank reactor, and followed by measurement of the activity of samples taken at regular time intervals, the relationship between operating time and the decrease in the enzyme activity was determined and also the half life ($t_{1/2}$) of the biocatalyst was determined.

Hydrolysis of Cholesterol in the Reactor

A continuous stirred tank reactor was used for the hydrolysis of cholesterol by using free and immobilized PLA₂. 3 ml of serum was mixed in the reactor for a few seconds and then an amount of 500 mg of immobilized PLA₂ or 0.56 ml of free enzyme (0.8 mg dry enzyme/ml) solution with the same protein content (0.325 mg) was added. The reactor was maintained at 37°C and stirred continuously with magnetic stirrer (150 rpm). The samples were withdrawn after constant time of incubation and the amount of cholesterol was determined as mentioned above.

RESULTS AND DISCUSSION

The immobilization of enzymes on inert and insoluble materials is currently a very active area of research because of their wide range of applications. Beside of this it offers several advantages over the use of soluble enzyme preparations including; easier separation of reaction products from the incubation mixture, the ability to recover and reuse of enzyme, stabilization of tertiary structure of enzyme and increased storage stability (Siso et al., 1997; Vaillant et al., 2000). Inorganic carriers employed in biotechnology are usually modified bifunctional organosilanes: in particular 3-aminopropyltriethoxysilane. Such modified carriers, especially porous glasses having controlled pore dimensions are widely applied in biotechnology, predominantly for enzyme immobilization (Janowski and Fischer, 1991).

In this work, pancreatic phospholipase A₂ (PLA₂) was immobilized on porous glass beads by covalent coupling. After the silanization of the glass beads, enzyme was coupled to the carrier by crosslinking with glutaraldehyde. We have also searched the usability of the immobilized PLA₂ for hypercholesterolemia treatment.

In previous works, phospholipases (PLA₂) from several sources have been immobilized by different methods: PLA₂ from cobra venom (*Naja naja*) was immobilized on the acrylic polymer Eupergit C (Madoery and Fidelio, 2001), on chitosan beads (Chen and Chen, 1998), on to agarose beads (Ferreira et al., 1993), from bee venom upon DEAE-Sephadex (Madoery et al., 1995), from porcine pancreas in alginate-silicate sol gel matrix (Kim et al., 2001) and etc. The porous glass-immobilized PLA₂ retained 21% of original activity. The immobilization results are summarized in Table 1.

Some parameters affecting to the enzyme activity (pH and temperature) and operational stability were investigated. The activities of free and immobilized enzymes were determined at different pH values. The optimum

Table 1. Immobilization of PLA₂ on porous glass beads.

Free enzyme			Immobilized enzyme				
Activity (unit)	Protein (mg)	Specific activity (unit/mg)	Bound protein (mg)	Bound protein (%)	Activity (unit)	Specific activity (unit/mg)	Activity yield (%)
1.16	1.45	0.8	0.65	44.8	0.11	0.17	21.3



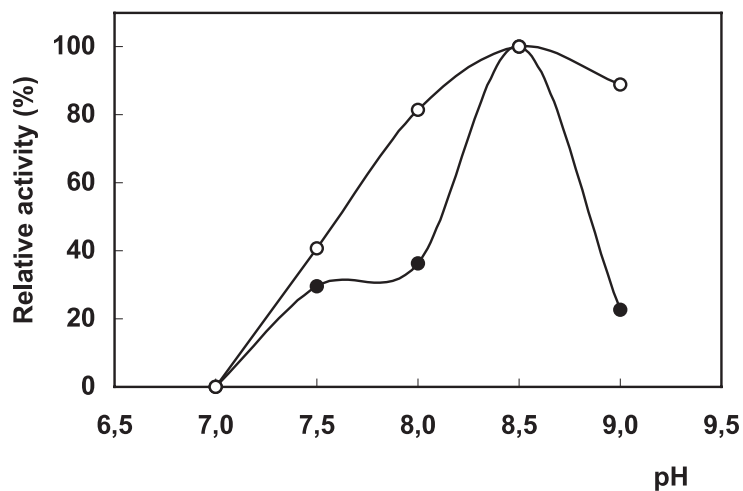


Figure 2. The effect of pH on the activity of free (○) and immobilized (●) PLA₂.

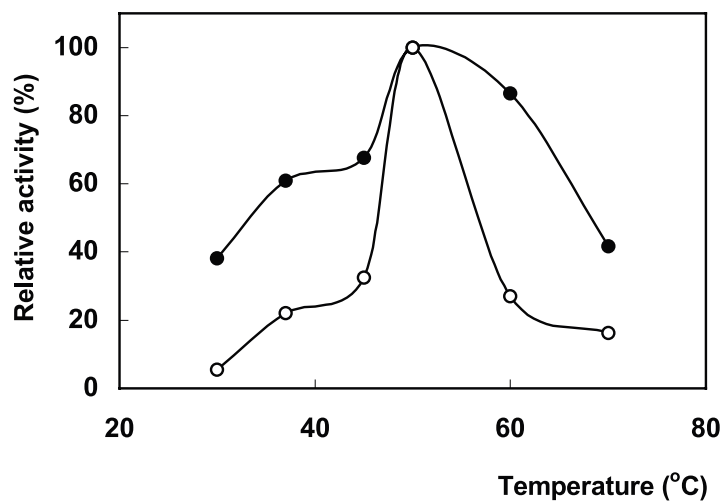


Figure 3. The effect of temperature on the activity of free (○) and immobilized (●) PLA₂.

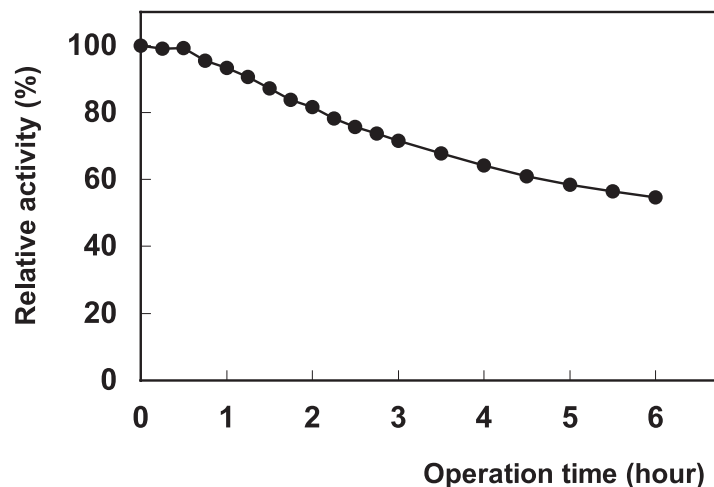


Figure 4. Operational stability of PLA₂ immobilized on porous glass.

pH values were determined from the graph of pH plotted against the percentage of relative activity (Figure 2). As is seen from the Figure 2, the optimum pH of the immobilized enzyme was pH 8.5. There is no shift from the pH optima of the free enzyme. The effect of temperature on the activity of the free and immobilized enzymes is shown in Figure 3. The maximum activity was observed at 50°C for both forms of PLA₂. However, the immobilized enzyme has a little broader temperature range than that of the free form. The immobilized PLA₂ was less sensitive to temperature changes compared to soluble enzyme. It has been reported that immobilized enzyme molecules could be preserved from conformational alterations induced by pH and temperature changes by a protective effect of the support (Madoery et al., 1995).

When comparing the catalysts intended for preparative or industrial use, characterization of their operational stabilities is very important. The operational stability of PLA₂ for lecithin is very good. Figure 4 shows that the immobilized enzyme lost about nearly 40% of its activity within 6 h and the operational half life of the enzyme was calculated as 6.9 h by using the formula given as;

$$t_{1/2} = 0.693/k_D \quad k_D = 2.303/t \times \log(A_0/A)$$

where t is the operation time, k_D is decay constant, A_0 and A are the enzymatic activities at the beginning and t time.



Previous approaches utilizing immobilized enzyme therapeutically caused either degradation of a toxin or its conversion to a nontoxic substance. Here, the immobilized enzyme modifies an existing molecule in the blood so that the body's own metabolic process can more easily eliminate it. The process of LDL removal by PLA₂ consists of two main steps: modification of LDL particles by enzymatic hydrolysis of the phospholipids on the surface of LDL to lysophosphatidylcholine and the enhanced uptake of the modified LDL by the liver.

To test the biomedical application of the immobilized PLA₂, we have carried out preliminary *in vitro* bioreactor study for lowering plasma cholesterol concentration in hypercholesterolemic humans. In treated serum a significant decrease in total cholesterol concentration was observed after 6 h treatment using a continuous stirred reactor containing immobilized PLA₂. The initial serum cholesterol concentration was 5.6 mmol/l (216 mg/dl). After the enzyme treatment, the cholesterol concentration decreased 17.7% as 4.61 mmol/l (178 mg/dl). In control experiments, with unmodified porous glass beads there was no change of cholesterol concentration.

In conclusion, the proposed immobilization procedure is very simple to carry out. The immobilized enzyme also showed good properties that are very important parameters in applications. Further optimization of this covalent immobilization process should lead to an immobilized PLA₂ system of great efficiency and useful for biotechnology processes or biomedical applications such as hypercholesterolemia treatment.

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