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Preparation and characterization of immobilized phospholipase A_2 on chitosan beads for lowering serum cholesterol concentration

Jyh-Ping Chen *, Jong-Yeh Chen

Department of Chemical Engineering, Chang Gung University, Kwei-San, Taoyuan 333, Taiwan, ROC

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Abstract

Phospholipase A_2 (PLA_2) from cobra venom, which can hydrolyze the S_N2 ester bond of 1,2-diacylphosphatides, was immobilized by covalent binding to porous chitosan beads. Immobilization has to be carried out by using the carboxylic groups instead of the amine groups of the enzyme to get reasonable activity retention (higher than 50%). The effects of amount of activating reagent EDC and enzyme loading during the immobilization step were investigated. Since EDC could modify important Asp groups in the enzyme, the EDC/enzyme weight ratio should be less than 10. Although the activity retention of immobilized enzyme increased with enzyme/bead weight ratio, this ratio should be kept to a minimum at 1×10^{-3} to optimize coupling yield of enzyme activity and reduce internal diffusion resistance. The kinetic properties and stability of the immobilized enzyme were determined. The immobilized PLA_2 was packed into a column to hydrolyze phospholipid in a circulating packed-bed reactor. The flow rate of the substrate solution should be set at 37.5 cm/min (superficial velocity) to eliminate external diffusion resistance, under which condition the column reactor could be reused up to 10 times with less than 20% loss of activity. Since enzymatic hydrolysis of phospholipid on low density lipoprotein (LDL) particle surface with PLA_2 could result in faster plasma clearance of the modified LDL particles, an in vitro bioreactor containing immobilized PLA_2 should be able to lower serum cholesterol concentration. A significant decrease in total serum cholesterol concentration in hypercholesterolemic rabbits was observed after 90-min treatment.

Keywords: Phospholipase A_2; Phospholipids; Chitosan; Low-density lipoprotein

1. Introduction

Phospholipase A_2 (PLA_2; EC3.1.1.4) is a lipolytic enzyme that hydrolyzes the ester bond in position 2 of phosphatidylglycerols. It is present in most animal and human tissues, and abundant in the mammalian pancreas and in the venom of several snakes and bees. Low density lipoprotein (LDL) particle is the major cholesterol carrier in the plasma with a spherical shape and with a diameter in the range of 20 to 25 nm. Enzymatic modification of LDL particle with PLA_2, by enzymatic hydrolysis of phospholipid on the particle surface, does not significantly change the basic structural features of the parti-

* Corresponding author: Fax: +886-3-3283031; e-mail: jpchen@cguaplo.cgu.edu.tw

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cle, including solubility [1]. Plasma clearance of PLA_2-modified human LDL was accelerated, up to 17 times faster than that of native human LDL in hypercholesterolemic rabbits, with liver being the main removal sites [2].

Several studies have established that the higher the total plasma cholesterol and LDL-cholesterol levels, the greater risk for coronary heart disease [3]. Drug therapy, diet control, and direct removal of LDL from the blood (plasmapheresis) can reduce plasma cholesterol. However, drug therapy may have side effects to limit its use. For example, drugs that decrease cholesterol synthesis, such as lovastatin, may cause liver and muscle injuries and fetal abnormalities. On the other hand, the success of diet control depends heavily on continued and sometimes unpleasant cooperation from patients. Moreover, many familial hypercholesterolemic patients, an autosomal disorder characterized by high levels of LDL–cholesterol in plasma, are resistant to diet and drug therapy. Plasmapheresis involves direct removal of patient's high cholesterol plasma and replacement with donor albumin. Although a successful therapy, plasmapheresis is expensive and removes a wide range of important proteins in addition to LDL [4]. Extracorporeal treatment for removal of LDL by adsorption to particles packed in columns can be accomplished with heparin-polyhydroxyethylmethacrylate beads [5], dextran sulfate–cellulose [6], or anti-LDL antibody–agarose [7]. However, the limited capacity of the adsorbents and the problems of fluid replacement and column regeneration make these techniques cumbersome and very expensive.

A promising and interesting route for treating high total plasma cholesterol and LDL–cholesterol levels is to use an extracorporeal enzyme bioreactor [2]. The bioreactor contains immobilized PLA_2 to modify plasma LDL, so that the modified LDL may be removed by an individual's own metabolic processes. A extracorporeal circuit was used to divert blood from hypercholesterolemic rabbit into a plasma separator. Red blood cells were diverted and keep isolated from PLA_2 to prevent hemolysis. The separated plasma was subsequently passed through the bioreactor containing immobilized PLA_2 where enzymatic modification took place. At the end of this treatment, the plasma was reconstituted with the blood cells and returned to the animal. Blood samples at the end of treatment revealed no significant net change in erythrocyte counts, leukocyte counts, platelet counts, or hematocrit. The concentrations of albumin, uric acid, bilirubin, and liver enzymes remained within normal range. Plasma levels of free hemoglobin were < 0.2 g/dl. The level of high-density lipoproteins showed no significant change with treatment [2]. Since this technique does not require any fluid replacement and adsorbent regeneration, it offers a potential safe approach for lowering cholesterol and LDL levels in blood.

In this study, we use porous chitosan beads as matrix for immobilization of PLA_2. Chitosan beads have a very narrow pore size distribution and shows excellent blood biocompatibility as derivatives of chitosan have been frequently used for biomedical purposes. The macro-porous structure of the matrix also provides good diffusivity of the LDL particles. The properties of the immobilized enzyme were characterized in batch and in a continuous packed-bed reactor. Its use in lowering plasma cholesterol concentration in test animals was also demonstrated.

2. Materials and methods

2.1. Materials

Porous chitosan beads (CHITOPEARL) carrying primary amine groups were obtained from Fuji Spinning, Tokyo, Japan. The particle diameter was between 800 to 1200 μm with an average pore diameter of 5 μm. PLA_2 from the venom of the cobra *Naja naja* was obtained from Sigma (St. Louis, MO, USA). Other reagents were of analytical grades and purchased from Sigma or Merck.

2.2. Enzyme immobilization

Chitosan beads-bearing reactive amino groups were used for immobilization of PLA_2. The amino groups were attached to the beads via space arms six carbon atoms long. The covalent immobilization of PLA_2 to CHITOPEARL beads involves the formation of an amide bond between carboxylic groups on the enzyme and the amine groups on the beads. 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) was used to activate carboxylic groups on PLA_2 in a reaction that was enhanced with *N*-hydroxysulfosuccinimide (sulfo-NHS) [8]. An amount of 1 mg of PLA_2 and 2.5 mg of L-α-lysophosphatidylcholine from egg (LPC) (a micellar substrate analogue to improve activity retention) were added to 1 g chitosan beads in 15 ml coupling buffer (20 mM 3-[*N*-morpholino]-2-hydroxypropanesulfonic acid (MOPSO) and 0.2 M $CaCl_2$, pH 6.9). An amount of 2.4 mg of sulfo-NHS and EDC solubilized in 1 ml coupling buffer, were added to the above mixture, maintaining pH 6.9. The mixture was left on an orbital mixer for 12 h at 25°C.

The mixture was filtered through a sintered funnel and washed as follows. Two washes of 10 ml coupling buffer; two washes of 10 ml 0.1 M $NaHCO_3$, 1 M NaCl, pH 8.0 solution; two washes of 10 ml 100 mM sodium acetate, pH 5.5 solution; two washes of 10 ml 1 mM sodium tetraborate, 25 mM $CaCl_2$, pH 8.0 solution. The resulting beads were then dried in a freeze drier (EYELA FD-5N) for 6 h and stores at -20°C. The amount of enzyme immobilized was measured based on the amount of PLA_2 remaining in the wash solutions as described above. This was accomplished using Micro BCA Protein Assay Reagent (Pierce) for colorimetric determination of proteins.

2.3. Enzyme activity assay

The assay used L-α-phosphatidylcholine dipalmitoyl (DiC16-PC) as micellar phospholipid substrate. The substrate solution was prepared by dissolving DiC16-PC to a concentration of 5 mM in a solution containing 1 mM sodium tetraborate, 10 mM $CaCl_2$, and 0.1 M NaCl, pH 8.0. To disperse the PC into micellar form, 118 μl of Triton X-100 was added to 10 ml of solution and subjected to ultrasonication (sonicator XL2020, Misonix) for 1 min. The enzyme reaction was carried out in a vessel surrounded by a heating jacket through which water at 37°C was circulated. The mixture was magnetically stirred to ensure uniform temperature and material distribution in the vessel. The free fatty acid that is produced in the enzymatic reaction was titrated with 5 mM NaOH using a Radiometer pH-stat apparatus consisting of a PHM290 pH controller and an ABU-901 Autoburette (Radiometer Copenhagen, France). Each mole of the substrate was hydrolyzed by the enzyme to yield 1 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 DiC16-PC per minute. Activities of enzyme were measured within the range of 0.4 to 3.5 μg of enzyme for both free and immobilized PLA_2, where activity showed linear dependence on protein concentration. The specific enzyme activities were calculated from the slopes of linear regression lines.

2.4. Hydrolysis of phospholipids in a circulating packed-bed reactor

A packed-bed reactor (Bio-Rad Econo-Column column, 0.5 cm $\times$ 5 cm) was used for hydrolysis of DiC16-PC to simulate the hydrolysis of phospholipids on LDL surface. The apparatus was set up as shown in Fig. 1. An amount of 40 mg (dry weight) of chitosan beads was swelled in borate buffer (pH 8.0) and packed in the reactor. The double-jacked vessel contains 15 ml substrate solution (5 mM DiC16-PC in pH 8.0 borate buffer as prepared above). The substrate solution was circulated through the reactor by a peristaltic pump at different flow

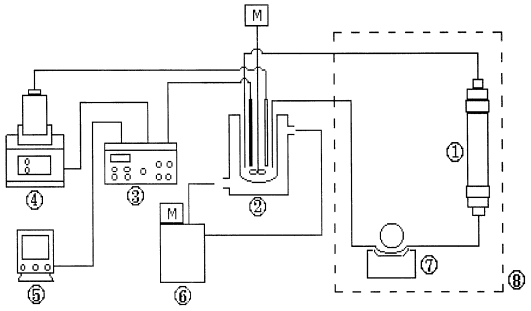

Fig. 1. Schematic diagram of the circulating packed-bed reactor for hydrolysis of phospholipid DiC16-PC with immobilized PLA_2. (1) packed-bed reactor; (2) double-jacked vessel; (3) pH controller; (4) autoburette; (5) personal computer; (6) constant-temperature water bath; (7) peristaltic pump; (8) constant-temperature incubator.

rates from 0.5 to 18 ml/min. The reactor was placed in a constant-temperature chamber at 37°C where hydrolysis reaction occurs. Aliquots of 25 mM NaOH was added to the double-jacked vessel (at 37°C) by an autoburette controlled by the pH controller to maintain the pH at 8.0. The volume of alkaline solution added was recorded for 1 h. After the hydrolysis reaction, the column was washed with 200 ml of DI water at the same flow rate and followed by borate buffer (pH 8.0) to start the next reaction.

2.5. In vitro bioreactor study

The procedure and circuit for extracorporeal treatment of Watanabe Heritable Hyperlipidemic rabbits with immobilized PLA_2 is similar to that as described by Labeque et al. [2]. The circuit consisted of a plasma separator, a water-jacked column (Bio-Rad Jacketed Econo-Column column, 1 cm × 15 cm) as the reactor, and two peristaltic pumps. Chitosan beads (as controls) or chitosan beads containing 100 U of immobilized PLA_2 were packed separately into two columns with 37°C water circulating through the outer jackets. The blood of the test animal was pumped through the plasma separator at 7.5 ml/min, and filtered plasma was perfused through the reactor. Treated plasma and blood cells were recombined and returned to the animals. Blood samples were withdrawn from a sampling port after 90-min treatment. The plasma cholesterol concentrations were measured in the clinical laboratory of Chang Gung Memorial Hospital.

3. Results and discussion

3.1. Enzyme immobilization

We have first tried to immobilize PLA_2 to chitosan beads through the protein's amine groups. However, the immobilized enzyme showed a severe loss of enzyme activity. Addition of micellar protectants LPC strongly inhibited the coupling reaction and had little effect on activity retention. Less than 13% activity retention was observed, indicating that important lysine groups of the enzyme were affected. This is consistent with a previous result, immobilization of *N. naja naja* PLA_2 through amine groups to tresyl chloride-activated Sepharose leads to a drastic loss of enzyme activity, less than 10% of the soluble form [9]. Previous chemical modification studies have revealed the importance of certain amine groups in snake and pancreatic PLA_2. *N. melanoleuca* PLA_2 was modified with 4-chloro-3,5-dinitrobenzoate which attacks almost specifically the Lys 6 residue. This residue is a conserved residue in all cobra venom enzymes [10]. The modified form had practically no activity. Other studies have shown that manoalogue also modifies lysine residue in cobra venom enzyme, which results in 70–80% loss of enzyme activity [11]. The crystal structure of *N. naja atra* PLA_2 confirmed that Lys 6 is part of the binding site of the substrate [12]. For rattlesnake *C. atrox* PLA_2, a similar situation was also reported, the enzyme covalently attached to agarose beads via amine groups led to an 80% loss of activity as compared with the soluble form. In contrast, enzyme immobilized via carboxylic groups was two to three folds more active than the one

Table 1
Effects of EDC amount added during the immobilization step on the activity retention of immobilized PLA_2

EDC/PLA_2 (w/w)	Specific activity (U/mg enzyme)
5	1310 ± 60
10	1301 ± 83
20	924 ± 20
30	762 ± 56
40	424 ± 23
50	245 ± 17

One milligram PLA_2, PLA/bead $= 1 \times 10^{-3}$ (w/w).
Sulfo-NHS/$PLA_2 = 2.4$ (w/w).
LPC/$PLA_2 = 2.5$ (w/w).
Specific activity of free enzyme = 2100 U/mg.

coupled through amine groups [13]. It is believed that covalent binding to support involves amine groups that are important for catalysis or maintaining protein structure.

Enzyme immobilization thus needs to be carried out by using the carboxylic groups of PLA_2. The amount of sulfo-NHS and LPC used in the immobilization protocol were proportional to the amount of PLA_2 used, to keep the ratios of sulfo-NHS/PLA_2 and LPC/PLA_2 at 2.4 and 2.5 (w/w), respectively. The weight ratios of enzyme to bead and EDC to enzyme were varied from 1 to 3 and 5 to 50, respectively. The efficiency of PLA_2 immobilization are shown in Tables 1 and 2 as specific activity of the immobilized enzyme vs. those ratios used in the immobilization reaction. The activity of the immobilized enzyme decreased with the amount of EDC used as the EDC/enzyme ratio exceeded 10. The immobilization method used the carbodiimide method with the help of condensing reagent EDC to form stable amide bonds between the enzyme and the carrier. EDC reacts with the carboxyl groups of the enzyme to form an active and unstable *o*-acylurea derivative, which subsequently can rearrange as an acyl urea or react with the primary amine groups of the support to yield the immobilized enzyme. Immobilization of PLA_2 through carboxylic groups results in a better activity retention than through amine groups of the enzyme [14]. PLA_2 have two conserved Asp residues involved in catalysis; Asp 49 interacts with the cofactor Ca^{2+} and Asp 99 is a residue of the catalytic domain [15,16]. Few studies were done on chemical modification of carboxylic groups in PLA_2s, except for the ones that determined the role of those two residues. Fleer et al. [15] have shown that, after extensive reaction of bovine pancreatic PLA_2 with EDC at pH 5.5, all but Asp 39 and Asp 99 carboxylic groups were modified; there was no residual activity. In the presence of Ca^{2+}, Asp 49 remained unmodified and the enzyme had 15% residual activity [15]. In order to avoid such a large degree of inactivation, immobilization were carried out with considerably milder conditions: low cabodiimide concentration, pH of reaction raised to pH 6.9, calcium and micellar substrate analogues (LPC) always present in saturating concentrations. Significant higher activity retention resulted in those cases with values generally higher than 50% (Table 1). The uncoupled enzyme collected in the washing buffers had the same

Table 2
Effects of enzyme loading during the immobilization step on the activity retention, coupling yield, and effectiveness factor of immobilized PLA_2

PLA_2/bead $\times 10^3$ (w/w)	Specific activity (U/mg enzyme)	Coupling yield of enzyme activity (%)	Effectiveness factor
1.0	1310 ± 60	44.3 ± 3.1	0.88
1.5	1414 ± 88	34.1 ± 2.2	0.69
2.0	1670 ± 63	31.4 ± 1.7	0.53
2.5	1801 ± 77	28.2 ± 1.2	0.35
3.0	1924 ± 12	25.0 ± 1.9	0.22

One gram bead, EDC/$PLA_2 = 5$ (w/w).
Sulfo-NHS/$PLA_2 = 2.4$ (w/w).
LPC/$PLA_2 = 2.5$ (w/w).
Specific activity of free enzyme = 2100 U/mg.

extent of inactivation as support-bound enzyme. This suggests that the activity loss upon immobilization were not due to the immobilization process itself but rather due to modification by EDC of catalytically important carboxylic groups.

The specific activity of the immobilized enzyme increased with the enzyme/bead ratio (or enzyme loading for fixed amount of bead) used in the immobilization reaction (Table 2). However, if based on the coupling yield of enzyme activity (enzyme activity immobilized/enzyme activity originally put in), the percentage of enzyme activity immobilized decreased sharply with increasing enzyme loading. As PLA_2 is rather expensive, we chose to optimize the enzyme to bead ratio based on the highest activity yield, which occurs at a value of 1.0×10^{-3} (w/w). Immobilized PLA_2 prepared with sulfo-NHS/$PLA_2 = 2.4$ (w/w), LPC/$PLA_2 =$ 2.5 (w/w), EDC/$PLA_2 = 5$ (w/w), and PLA_2/bead $= 1 \times 10^{-3}$ (w/w) were prepared and used in the following studies.

3.2. Enzyme properties and hydrolysis of phospholipids in a circulating pack-bed reactor

The specific activities of free and immobilized PLA_2 at different substrate concentrations were measured using the titration method with DiC16-PC as the substrate. Lineweaver–Burk plots were obtained as shown in Fig. 2. The kinetic parameters were calculated from the plots using a linear least square fit. The K_m values are 10.35 and 9.23 mM, and the V_{max} values are 3644 (U/mg enzyme) and 2729 (U/mg enzyme), respectively, for free and immobilized PLA_2. The decrease in K_m value indicates the immobilized enzyme has higher affinity for PC than the free enzyme.

For long-term storage stability of the immobilized enzyme, no significant loss of enzyme activity was observed when freeze-dried beads containing the enzyme was stored at -20°C for 4 months. To test the long-term stability of

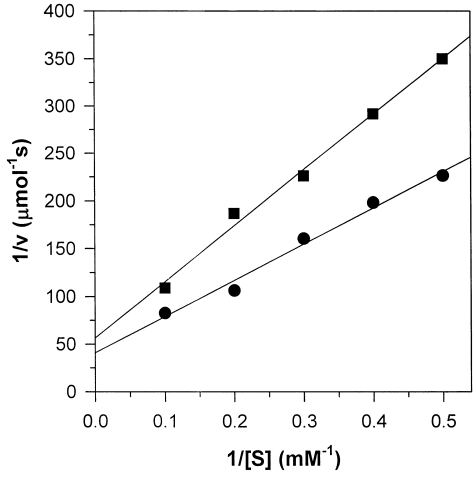

Fig. 2. Kinetics of DiC16-PC hydrolysis by free and immobilized PLA_2 at 37°C in pH 8.0 buffer. The lines are best-fits of the data. Each activity measurement is an average of three, having a standard deviation of less than 6%. (●) free enzyme, (■) immobilized enzyme.

immobilized PLA_2 at body temperature, studies were performed to detect the effect of incubation time on PLA_2 activity. The immobilized enzyme was incubated in vitro at 37°C in pH 8.0 borate buffer. The enzyme activity showed an exponential decay with a half-life of enzyme activity of 26 days.

For determining the internal diffusion effects of the immobilized enzyme, effectiveness factors were determined for beads prepared with different enzyme loading (Table 2). The effectiveness factor was defined as the apparent enzyme activity of 10 mg intact enzyme-containing beads to that of 10-mg broken enzyme-containing beads. The broken bead was prepared by carefully grinding the particles in an ice bath to destroy the structure of the beads. The effectiveness factor increased with decreasing enzyme loading, where the internal diffusion effect became less important on overall reaction rate observed. The highest effectiveness factor being 0.88. Previous studies immobilizing a similar enzyme to agarose beads did not observe diffusion limitations with short-chain lecithin (DiC7-PC) as substrate at low protein loading [9,14]. With the long-chain lecithin as substrate, internal diffusion limitation was observed here within

the enzyme loading studied. Theoretically, this limitation may be reduced or totally eliminated with even lower enzyme loading.

To simulate the in vitro bioreactor study, a circulating packed-bed reactor was set up as shown in Fig. 1 for hydrolysis of the phospholipid substrate. NaOH was added to neutralize the fatty acid released during the course of enzymatic hydrolysis of DiC16-PC. To monitor the progress of the reaction, the weight of NaOH solution added to maintain a constant pH in the double-jacked reaction vessel was recorded as a function of time at different flow rates of the substrate solution. As shown in Fig. 3, the rate of NaOH addition increased with increasing flow rate, and the curve levelled off quickly at high flow rates, corresponding to total consumption of substrate. The activities of the enzyme-containing beads were calculated from the slopes of the initial linear region and reported in the insert of Fig. 3 as relative activity. The apparent activity of immobilized PLA_2 increased from 0.5 to 7.5 ml/min (superficial velocity from 2.5 to 37.5 cm/min) and remained constant thereafter. This behavior was caused by external diffusion resistance as it is known that higher linear flow rate of solution through a column of packed particles can reduce the stagnant film around the beads and increase the mass transfer of substrate into the beads. The external diffusion resistance is not important after the linear flow rate increased above 7.5 ml/min. To test the reusability of column, the column reactor was operated up to 10 times for each 60-min cycle at 7.5 ml/min flow rate. The activity was calculated for each cycle from the linear region as before. The beads showed 83% of its original activity during the tenth cycle. Based on those results, we concluded that this column reactor would be suitable for repeated hydrolysis of LDL in the serum of a hypercholesterolemic patient with storage of the reactor in 4°C environment between uses to preserve the enzyme activity.

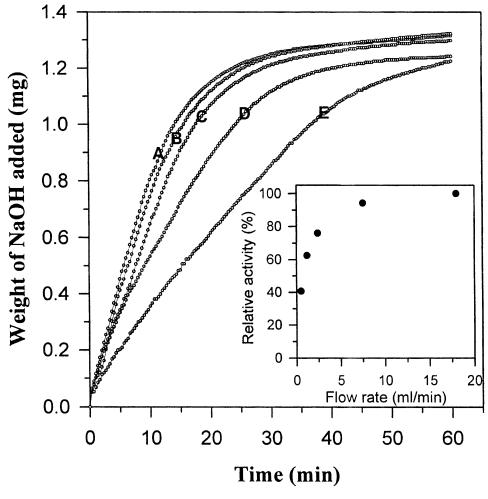

Fig. 3. Hydrolysis of DiC16-PC by immobilized PLA_2 at 37°C in pH 8.0 buffer in a circulating packed-bed reactor at different substrate flow rates. The progress of the reaction was monitored by following the total weight of 25 mM NaOH solution added to the double-jacked vessel for neutralizing the fatty acids liberated in the reaction. The activity of PLA_2 in the reactor was calculated from the slope of the initial linear region of each curve. The inset shows the relative activity under different flow conditions. Flow rate of substrate solution: (A) 18 ml/min; (B) 7.5 ml/min; (C) 2.4 ml/min; (D) 1.2 ml/min; (E) 0.5 ml/min.

3.2.1. In vitro bioreactor study

Previous approaches utilizing immobilized enzymes therapeutically caused either degradation of a toxin or its conversion to a nontoxic substance [17]. 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 PLA_2 consists of two main steps: modification of LDL particles by enzymatic hydrolysis of the phospholipids (phosphatidylcholine) on the surface of LDL to lysophosphatidylcholin (LPC), and the enhanced uptake of the modified LDL by the liver.

To test the biomedical applications of the immobilized PLA_2, we have carried out preliminary in vitro bioreactor study for lowering plasma cholesterol concentration in hypercholesterolemic rabbits. In all treated animals ($n = 6$), a significant decrease in total plasma cholesterol concentration was observed after 90-min treatment. The plasma cholesterol concen-

tration decreased 24% ± 8% with initial plasma cholesterol concentrations in the range of 230–600 mg/dl. The maximum decrease percentage attained was 32%. In control experiments with no PLA_2 attached to the beads, no change of plasma cholesterol concentration was observed ($n = 3,\ P < 0.01$).

One of the major safety concerns surrounding devices utilizing immobilized enzymes for biomedical purposes is the extent to which the enzyme may be leached out of the device into circulation. PLA_2 leaching could lead to hemolysis as the enzyme modifies phospholipids in the red blood cell membrane. The possible leaching of PLA_2 in the extracorporeal circuit was investigated by measuring PLA_2 enzyme activity in blood samples withdrawn from the sampling port during the whole course of the treatment. No enzyme activity could be detected in all samples collected.

Acknowledgements

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