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Expression and purification of lipoprotein-associated phospholipase A$_2$, a key enzyme involved in atherosclerosis[1]

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Key words

Abstract

Aim: To express and purify lipoprotein-associated phospholipase A$_2$ (Lp-PLA$_2$), and to establish a screening model for Lp-PLA$_2$ inhibitors using the expressed Lp-PLA$_2$. **Methods:** We cloned the full-length cDNA of Lp-PLA$_2$ from differentiated THP-1 cells, and subcloned the cDNA into the baculovirus transfer vector pFastBac1. In addition, we introduced an N-terminal Kozak sequence for high-level translation initiation and a C-terminal sequence of 6 histidine residues for purification. The fusion enzyme was expressed in Sf9 insect cells and purified by Ni^{2+} affinity chromatography. Recombinant Lp-PLA$_2$ activity was measured using [^{3}H]PAF as a substrate, and we examined the enzyme activity of recombinant Lp-PLA$_2$ pretreated with the known Lp-PLA$_2$ inhibitor SB435495. **Results:** We successfully cloned the full-length Lp-PLA$_2$ gene from differentiated THP-1 cells. The fusion enzyme was expressed in Sf9 insect cells at a high level and purified efficiently through a 2-step procedure. The recombinant Lp-PLA$_2$ activity was measured using [^{3}H]PAF as a substrate, and proved to be enzymatically active. Lp-PLA$_2$ inhibitor SB435495 produced a good inhibition curve for inhibition of recombinant Lp-PLA$_2$ with an IC$_{50}$ of 57±1 µmol/L. **Conclusion:** We expressed and purified Lp-PLA$_2$ at a high level in insect cell-baculovirus expression system. The yield ratio was much greater than that obtained from human plasma and we established a screening model for Lp-PLA$_2$ inhibitors using the expressed Lp-PLA$_2$.

Introduction

Atherosclerosis is the major cause of many cardiovascular conditions and it is, primarily, a chronic inflammatory disease. Lipoprotein-associated phospholipase A$_2$ (Lp-PLA$_2$), also known as a platelet activating factor (PAF) acetylhydrolase, plays a key role in atherogenesis through the release of pro-inflammatory signals[1]. The Lp-PLA$_2$ protein, which consists of 441 amino acids and has a predicted molecular weight of 45 kDa[2], is a member of the phospholipase A$_2$ superfamily and belongs to the VII phospholipase A$_2$ subgroup. The protein is produced mainly by monocytes, macrophages, T-lymphocytes, and mast cells, and can hydrolyze PAF, PAF-like phospholipids and polar-modified phosphatidylcholines[3]. The majority of Lp-PLA$_2$

in human plasma (approximately 70%) is associated with low density lipoprotein (LDL), and the remainder is associated with high density lipoprotein (HDL). Lp-PLA$_2$ can hydrolyze oxidized LDL to generate lysophosphatidylcholine (lyso-PC) and oxidized fatty acids; both of these hydrolysis products are pro-inflammatory factors that have pro-atherosclerotic effects. The increased levels of lyso-PC in oxidized LDL can be completely attributed to Lp-PLA$_2$[3,4], the expression of which is regulated by inflammatory mediators. Lp-PLA$_2$ activity is upregulated in atherosclerotic lesions, especially in complex plaques, and Lp-PLA$_2$ appears to be an independent predictor of coronary artery disease (CAD) status[5,6]. It has been proposed that high plasma Lp-PLA$_2$ levels should be viewed as a potential risk factor for CAD, and that Lp-PLA$_2$ monitoring could provide information comple-

mentary to traditional lipoprotein measurements[7–9]. As such, Lp-PLA$_2$ has been proposed as an important target molecule for the design of new treatment strategies for coronary heart disease (CHD)[10–12].

There is a clear need for inhibitors of Lp-PLA$_2$ to more fully understand the role of this lipase in the atherosclerotic process. Use of Lp-PLA$_2$ inhibitors is a new method separated from current lipid-lowering approaches for atherosclerosis therapy[13,14]. However, the quantity of Lp-PLA$_2$ that can be purified from human serum is limited[15], so it is necessary to express this protein through a proper expression system for the study of Lp-PLA$_2$ and its inhibitors. The identification of Lp-PLA$_2$ inhibitors would aid our evaluation of the role of this enzyme in atherosclerosis, and the inhibitors can be potentially developed into new drugs for treating atherosclerosis. In the present paper, we report cloning of the Lp-PLA$_2$ gene from THP-1 cells, and the expression and purification of Lp-PLA$_2$. The recombinant Lp-PLA2 was enzymatically active and it could be used to establish a screening model for Lp-PLA2 inhibitors.

Materials and methods

Materials RPMI-1640 medium and Grace's insect cell culture medium were purchased from Gibco (Carlsbad, USA). Sf-900 II serum-free medium was obtained from Invitrogen (Carlsbad, USA). Phorbol 12-myristate 13-acetate (PMA) was obtained from BioMol (Plymouth Meeting, USA). The Trizol reagent, Cellfectin reagent, and baculovirus expression vector system were purchased from Invitrogen (Carlsbad, USA). The M-MLV reverse transcriptase, restriction endonucleases, and T4-DNA ligase were obtained from Promega (Madison, USA). The oligonucleotides were synthesized by Sangon (Shanghai, China). The Pyrobest DNA polymerase was obtained from TaKaRa (Dalian, China). The Lp-PLA$_2$ antibody was purchased from Cayman (Ann Arbor, USA). 1-*O*-alkyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine [(^{3}H-acetyl)PAF] was purchased from PerkinElmer (Wellesley, USA). The Ni-NTA His·Bind resin was obtained from Novagen (Nottingham, UK), and SB435495 was supplied by the Department of Medicinal Chemistry, Shanghai Institute of Materia Medica.

Gene cloning of Lp-PLA$_2$ from THP-1 cells THP-1 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS). The cells were seeded in 6-well plates at a density of 8×10^8 cells/L and incubated for 6 h, and then the cells were stimulated to differentiate with the addition of 81 nmol/L PMA. Forty-eight hours later, the differentiated THP-1 cells were collected and total RNA was extracted

using Trizol reagent. The total mRNA was reverse transcribed with 200 U M-MLV reverse transcriptase, 1 μL 0.5 g/L oligo d(T)$_{18}$-primer and 0.5 mmol/L dNTPs, and the resulting cDNA was isolated and amplified by polymerase chain reaction (PCR) with Lp-PLA$_2$-specific primers (sense, 5'-GCG C<u>GT CGA C</u>AG GGG GAC AGC GGC TGG TCG GAG GC-3'; antisense, 5'-GCG C<u>CT CGA G</u>CC TAA TTG TAT TTC TCT ATT CCT GA-3'; *Sal* I and *Xho* I sites underlined). The PCR program consisted of an initial denaturation at 95 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 56 °C for 45 s, then 72 °C for 2 min. In order to introduce a Kozak sequence for high-level translation initiation and a His$_6$-tag sequence for purification, the gene was purified and PCR amplified with primers: sense, 5'-CCG <u>GTC GAC</u> CGG <u>ACC ATG</u> GTG CCA CCC AAA TTG CAT-3'; antisense, 5'-CCG <u>CTC GAG</u> CGG TTA **GTG ATG GTG ATG GTG ATG** *GCC CTG AAA ATA CAG GTT TTC* ATT GTA TTT CTC TAT TCC TGA-3' (*Sal* I and *Xho* I sites is underlined and Kozak sequence is double underlined; bold represents the nucleotide sequence encoding 6 histidine residues and italics represents the TEV protease recognition site). The second PCR program consisted of an initial denaturation at 95 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 56 °C for 45 s and then 72 °C for 2 min.

Construction of donor vector The PCR product was electrophoresed in a 1.5% (*w/v*) agarose gel and purified with an agarose gel purification kit (Watson, Shanghai, China) in accordance with the manufacturer's instructions. The purified DNA fragment was digested with *Sal* I and *Xho* I, and cloned into the *Sal* I- and *Xho* I-digested pFastBac1 donor vector. The nucleotide sequence of the fragment cloned into the plasmids was confirmed by DNA sequencing.

Generation of recombinant baculovirus transfer vector The validated pFastBac1 plasmids were amplified and isolated from DH5α. We used 0.1–1 μg of this DNA to transform 100 μL of competent DH10BAC cells. Colonies containing the recombinant bacmid were white, and colonies containing the unaltered bacmid were blue. The white colonies were selected for isolation of recombinant bacmid DNA. The white candidate colonies were streaked on isopropyl-1-thio-*D*-galactopyranoside (IPTG) plates to ensure that they were truly white colonies. Recombinant bacmid DNA was isolated from bacterial transformants using standard procedures with a minor modification (all samples were centrifuged at 18 000×*g* instead of 14 000×*g*). The recombinant bacmid DNA was verified to be a correct transcript by PCR.

Generation of recombinant baculovirus Sf9 cells were grown in Grace's insect cell culture medium containing 10% FBS. The cells were transfected with recombinant bacmid using Cellfectin reagent as intermedia. Viral supernatant was

collected at 72 h post-transfection and the viruses were amplified by infecting Sf9 cells with the viral supernatant. The recombinant viruses were subjected to 2 rounds of amplifications in order to obtain a high-titer stock. All virus stocks were stored at -20 °C and protected from light to ensure maintenance of titer.

Expression of recombinant Lp-PLA$_2$ in insect cells Sf9 cells were grown in Grace's insect cell culture medium supplemented with 10% FBS at first, and then were adapted to grow in Gibco Sf-900 II serum-free medium at 27 °C. To examine the time course of Lp-PLA$_2$ expression, Sf9 cells (6×10^6/well) were seeded into a 6-well plate and grown in 2 mL SF900 II medium. The cells were infected with recombinant baculoviruses at a multiplicity of infection (MOI) of 0.1. Cells were harvested at different time points by 5 min centrifugation at $500 \times g$. For larger scale expression of (His)$_6$-Lp-PLA$_2$, Sf9 cells were cultured in 75 cm^2 flasks and infected with recombinant high-titer virus stock. The cells were harvested at 48–96 h post-infection. Harvested cells were washed twice with 0.1 mol/L phosphate buffer (pH 7.4). The cells were resuspended in 8 volumes of lysis buffer (50 mmol/L Tris, 300 mmol/L NaCl, 10 mmol/L imidazole, 10 mmol/L CHAPS, 1 mg/L antipain, 10 mg/L leupeptin, and 1 mg/L pepstatin A; pH 7.5) and sonicated on ice for 80 s. The lysate was centrifuged at 18 000×*g* for 30 min at 4 °C and the clear supernatant was collected and kept at -80 °C until further processing.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 5% polyacrylamide stacking gel and a 10% polyacrylamide separation gel. The proteins were visualized in the gels by staining with Coomassie brilliant blue R-250. For Western blot analysis, the proteins were transferred to nitrocellulose membranes and nonspecific binding was blocked with Tris-buffered saline (TBS; 100 mmol/L Tris-HCl, pH 7.5, 155 mmol/L NaCl) containing 3% albumin (bovine, fraction V; Amresco, Solon, USA). The nitrocellulose membrane was incubated with Lp-PLA$_2$ antibody (1:1000) for 2 h at room temperature, and then with horseradish peroxidase-conjugated anti-rabbit IgG (Calbiochem, San Diego, USA) for 2 h at room temperature. The immune complexes were detected and visualized using the SuperSignal West Pico Trial Kit (Pierce, Rockford, USA) according to the manufacturer's instructions.

Purification of recombinant Lp-PLA$_2$ All purification assays were performed at 0 °C, unless stated otherwise. The thawed supernatant was recentrifuged at 18 000×*g* for 30 min (to ensure that no residual cell debris was present) and the clear supernatant was removed to a fresh tube. Saturated ammonium sulfate was added slowly into the supernatant to obtain a stepwise increase in ammonium sulfate concentrations. The solution was put on ice overnight to allow the precipitation process to conclude, and then centrifuged to collect the protein precipitate. The protein precipitate was dissolved in lysis buffer (50 mmol/L Tris, 300 mmol/L NaCl, 10 mmol/L imidazole, 10 mmol/L CHAPS, 1 mg/L antipain, 10 mg/L leupeptin, and 1 mg/L pepstatin A; pH 7.5). The solution containing the soluble His$_6$-Lp-PLA$_2$ was incubated with 1.5 mL 50% (*v/v*) Ni-NTA His·Bind resin (equilibrated in lysis buffer) at 4 °C with gentle rocking for 1 h. The mixture was then loaded on a 10 mL column under gravity flow and the column was washed twice with 6 mL wash buffer (50 mmol/L NaH$_2$PO$_4$, pH 8.0, 300 mmol/L NaCl, and 20 mmol/L imidazole). The (His)$_6$-Lp-PLA$_2$ was eluted from the column with 4× 0.75 mL elution buffer (50 mmol/L NaH$_2$PO$_4$, pH 8.0, 300 mmol/L NaCl, and 250 mmol/L imidazole).

Enzyme assays Lp-PLA$_2$ activity was measured using [^{3}H]PAF as a substrate. All assays were performed at 37 °C in 50 mmol/L *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and 150 mmol/L NaCl, pH 7.4. Lp-PLA$_2$ was incubated with 20 nmol/L [^{3}H] PAF in a final volume of 200 μL for 10 min at 37 °C. The reaction was stopped by vortexing with 600 μL of CHCl$_3$/MeOH (2:1), and the CHCl$_3$ and aqueous layers were separated by a 15 min centrifugation at 12 080×*g*, and 250 μL of the upper aqueous layer was removed to a fresh tube and vortexed with 250 μL of CHCl$_3$. The upper aqueous layer was again removed to a fresh tube and the amount of liberated [^{3}H]acetate was determined by scintillation counting. The protein concentration of the fractions was measured using the Bradford's method[16] with minor modifications. Briefly, 10 μL of protein solution was added into 3 mL of protein reagent and mixed by vortexing. The absorbance at 595 nm was measured after 10 min in 3 mL cuvettes against a protein reagent blank. The concentration of protein was determined according to a standard curve constructed using bovine serum albumin.

To examine the inhibitory effects of various compounds on the enzyme, purified Lp-PLA$_2$ and the compounds were preincubated at 37 °C for 10 min before running the enzyme assay as described earlier.

Results

Gene cloning and generation of recombinant bacmid We used the THP-1 human acute monocytic leukemia cell line, and stimulated the cells to differentiate. The Lp-PLA$_2$ gene was successfully cloned from the cDNA library of differentiated THP-1 cells, whereas Lp-PLA$_2$ mRNA in non-stimulated

THP-1 cells was not detected by PCR. We engineered the Lp-PLA$_2$ gene to include a Kozak sequence for highly efficient translation initiation and a 6-His-tag sequence for purification. The fusion Lp-PLA$_2$ gene was subcloned into the pFastBac donor plasmid. The amplified clones were sequenced in their entirety and found to be identical to the human sequence in the National Institutes of Health GenBank database (GenBank accession no U24577). The reconstructed donor plasmid was transfected into DH10BAC cells and recombined with the bacmid in the DH10BAC cells. This procedure yielded approximately 20–80 white colonies per plate. We found that 10%–30% of the total were white colonies. Eight out of 50 white colonies were selected after the selection procedure, and the recombinant bacmid was extracted from the DH10BAC cells. PCR results confirmed the correct recombination of the bacmid with the donor plasmid.

High-level expression of recombinant Lp-PLA$_2$ in insect cells The recombinant bacmid was transfected into Sf9 cells and the baculovirus was amplified in the cells. The Sf9 cells were adapted in serum-free medium SF900-II, and the adapted Sf9 cells performed well in the SF900-II serum-free medium. We examined the effect of the Kozak sequence on the expression level of recombinant Lp-PLA$_2$, and found that the Kozak sequence had greatly increased the expression level of Lp-PLA$_2$. The recombinant Lp-PLA$_2$ was expressed at a high level (Figure 1). As measured by densitometry scanning, the amount of recombinant Lp-PLA$_2$ was up to 14.5% of the total cellular protein in the late stages of infection.
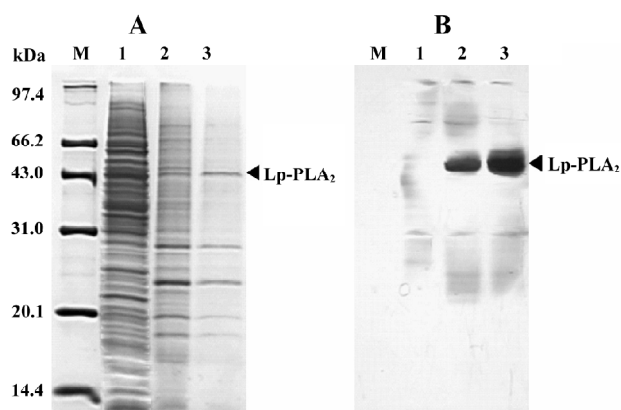

Figure 1. Infected cells were grown for 0 to 96 h at 27 °C, and cell proteins were extracted for analysis by SDS-PAGE and Western blotting. HPI, h post-infection. (A) SDS-PAGE analysis of Lp-PLA$_2$ expression in Sf9 cells. Lane M, molecular weight marker; lane 1, 24 HPI; lane 2, 48 HPI; lane 3, 72 HPI. (B) Western blot analysis of Lp-PLA$_2$ expression in Sf9 cells; the result was the same as from the SDS-PAGE gel. Lane 1, 24 HPI; lane 2, 48 HPI; lane 3, 72 HPI. Horizontal small arrows indicate the position of expressed Lp-PLA$_2$.

No Lp-PLA$_2$ was detected within the first 24 h post-infection (Figure 1B). To examine the time course of enzyme activity, cells were harvested at 0, 24, 48, 72, and 96 h after infection, and the enzyme activity of cell proteins was measured as described earlier. Our results revealed that the enzyme activity peaked 72 h after baculovirus infection (Figure 2).
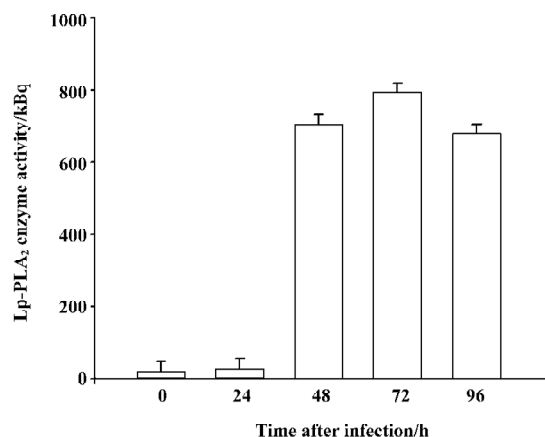

Figure 2. Time course of the enzyme activity level of expressed Lp-PLA$_2$ in Sf9 cells. Cells were harvested at the time points indicated, and the enzyme activity of cell lysis supernatant was determined using [^{3}H-acetyl]PAF as a substrate. The radioactivity of the supernatant (in kBq, as shown on the Y-axis) represented the enzyme activity of expressed Lp-PLA$_2$ in Sf9 cells. n=3. Mean±SD.

Purification of recombinant histidine-tagged Lp-PLA$_2$ The cell extraction proteins were first subjected to a stepwise precipitation with different ammonium sulfate saturations. We found that most of the recombinant protein was precipitated, and that only a few other proteins precipitated when the ammonium sulfate was at 20% saturation. After the recombinant proteins were first partially purified by ammonium sulfate precipitation, the precipitate was redissolved in the lysis buffer and purified through Ni-NTA His·Bind resin. The histidine-tagged Lp-PLA$_2$ protein was purified to near homogeneity after the purification process (Figure 3). The yield ratio was lower than 14.5% because some recombinant Lp-PLA$_2$ was lost in the purification process. The final yield obtained from insect cells was 0.54 mg per 20 mg cell proteins with at least 95% purity, and the yield ratio was 2.7%, which was much greater than that obtained from human plasma. The final yield obtained from human plasma was 7.0 mg per 6440 mg plasma proteins, and the yield ratio was 0.11%. The results of a typical purification are summarized in Table 1.

Inhibitive effect of SB435495 on recombinant Lp-PLA$_2$ Recombinant Lp-PLA$_2$ was first identified by Western blot-
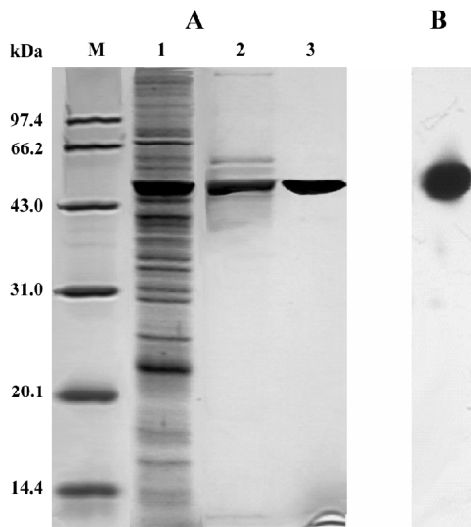
Figure 3. Purification of recombinant $(His)_6$-Lp-PLA$_2$ from baculovirus-infected Sf9 cells. (A) Representative samples were analyzed by SDS-PAGE and 10 μg protein was loaded in each lane. Lane M, molecular weight marker; lane 1, total proteins of infected Sf9 cells; lane 2, partially purified Lp-PLA$_2$ by ammonium sulfate precipitation; lane 3, purified Lp-PLA$_2$ by Ni^{2+} affinity chromatography. (B) Western blot analysis of the purified recombinant Lp-PLA$_2$.

ting using the highly specific anti-Lp-PLA$_2$ antibody (Figure 1B). The specific activity of $(His)_6$-Lp-PLA$_2$, using [^{3}H]PAF as substrate, was measured (Table 1). In order to further identify and examine the usefulness of our recombinant Lp-PLA$_2$ for screening Lp-PLA$_2$ inhibitors, we examined the enzyme activity of recombinant Lp-PLA$_2$ pretreated with the known Lp-PLA$_2$ inhibitor SB435495. The inhibitor produced a good inhibitory curve for the inhibition of recombinant Lp-PLA$_2$ (Figure 4) and it inhibited the recombinant enzyme with an IC$_{50}$ of 57±1 μmol/L. These results collectively indicate that our recombinant Lp-PLA$_2$ protein could be used to

Table 1. Purification of recombinant $(His)_6$-Lp-PLA$_2$ from infected Sf9 cells (result from one experiment with a 75 mL culture of $(His)_6$-Lp-PLA$_2$ infected Sf9 insect cells). A unit of enzyme activity was defined as the release of 1 nmol of [^{3}H]acetate per L reaction solution at 37 °C.

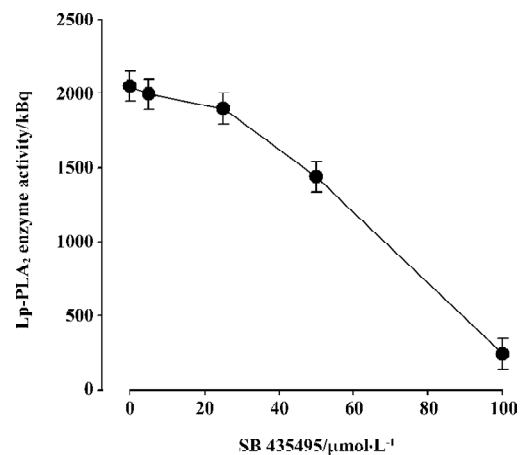| Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield activity (%)
Cell extract	20	10667	533	100
Ammonium sulfate	6.2	8583	1384	80.5
Ni^{2+}-NTA	0.54	2917	5401	27.3

Figure 4. Inhibitory curve for inhibition of recombinant Lp-PLA$_2$ by SB435495. The enzyme activity of 0.5 μg Lp-PLA$_2$ was measured following pretreatment with 0.05, 5, 25, 50, or 100 μmol/L SB435495. *n*=3. Mean±SD.

establish a screening model for Lp-PLA$_2$ inhibitors, and that SB435495 could be used as a positive control.

Discussion

Lp-PLA$_2$ is an inflammatory marker that has been identified as a novel therapy target for atherosclerosis[17–19]. Inhibition of Lp-PLA$_2$ has anti-atherogenic effects. However, the large-scale screening of Lp-PLA$_2$ inhibitors has proven to be difficult. Direct screening of plasma LDL may yield nonspecific inhibitors due to the presence of other phospholipase A$_2$ molecules, while purification of Lp-PLA$_2$ from plasma is complicated and produces a very low yield. Thus, we herein sought to develop a simple, large-scale method for producing recombinant Lp-PLA$_2$ suitable for inhibitor studies.

In the present study, we reported the cDNA cloning of Lp-PLA$_2$ from differentiated THP-1 cells. We engineered the N-terminal of the cDNA to possess a Kozak sequence for high-level translation initiation. We found that the Kozak sequence greatly increased the expression level of recombinant Lp-PLA$_2$ in Sf9 insect cells. The amount of recombinant Lp-PLA2 was up to 8.5% of the total cellular protein, and the inclusion of the Kozak sequence further increased this expression up to 14.5% of total cellular proteins. A very high expression level in insect cells was achieved. We also adapted the Sf9 cells in serum free SF900-II media to remove the interference of serum in enzyme assays.

The recombinant Lp-PLA$_2$ was constructed as a fusion protein with a 6-His tag at the C-terminus for convenient purification by affinity chromatography. The enzyme could

not be purified directly using the Ni-NTA His·Bind resin at first, so we partially purified the protein by ammonium sulfate precipitation before the affinity purification step. The enzyme was efficiently purified through this 2-step procedure. The enzyme activity of the recombinant Lp-PLA$_2$ was 115 µmol/min per mg, and it was at the same level of the activity of the enzyme purified from human plasma, which was 112 µmol/min per mg. The recombinant purified Lp-PLA$_2$ was enzymatically active, and the inhibitor SB435495 inhibited the enzyme activity. Thus, using our recombinant Lp-PLA$_2$, we could establish a screening system for Lp-PLA$_2$ inhibitors.

In conclusion, we reported gene cloning of Lp-PLA$_2$ from differentiated THP-1 cells. We demonstrated how to take advantage of recently developed molecular cloning technologies to successfully express and purify Lp-PLA$_2$ at a high level in the insect cell-baculovirus expression system. The yield ratio was much greater than that obtained from human plasma. We established a screening model for Lp-PLA$_2$ inhibitors, and the screening model would facilitate larger scale screening of Lp-PLA$_2$ inhibitors and discovery of more novel inhibitors. The recombinant Lp-PLA$_2$ and its novel inhibitors would aid further research regarding the role of this enzyme in atherosclerosis and the development of new drugs for atherosclerosis therapy.

Acknowledgements

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