Scientific paper

Bacterial Expression and Simple Purification of Human Group X Secretory Phospholipase A_2

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Abstract

Secreted group X phospholipase A_2 (sPLA$_2$-X) is one of the most effective mammalian PLA$_2$ enzymes at hydrolyzing plasma lipoproteins and phospholipids in the membranes of intact cells, due in particular to its relatively high binding affinity to zwitterionic phospholipid substrates, such as phosphatidylcholine. The products of its enzymatic activity, lysophospholipids and free fatty acids, especially arachidonic acid, are involved in various physiological and pathological processes and currently being studied intensively. In spite of numerous studies, the biological roles of sPLA$_2$-X have not been completely elucidated. With the aims of studying various cellular functions and designing effective enzyme inhibitors, we prepared a high amount of recombinant human sPLA$_2$-X. Here we describe an effective *Escherichia coli* expression system, together with an *in vitro* refolding and simple purification procedure, that yields up to 10 mg of mature human sPLA$_2$-X from a litre of culture. In contrast to the natural protein, the recombinant enzyme was produced in bacterial cells without the N-terminal propeptide, *i.e.* as a mature protein, and was not N-glycosylated. It however retained all the enzymatic properties for hydrolysis of vesicular substrates composed of either phosphatidylglycerol or phosphatidylcholine.

Keywords: Recombinant protein; inclusion bodies; refolding; isolation; enzymatic activity; phospholipid hydrolysis

1. Introduction

Mammalian secretory phospholipases A_2 (sPLA$_{2S}$) comprise a family of 10 enzymes that catalyse the hydrolysis of the *sn*-2 ester bond of phospholipids to produce a free fatty acid and lysophospholipid. sPLA$_2$s are small enzymes (14–16 kDa), possessing a His/Asp catalytic dyad in the active centre and a conserved Ca^{2+}-binding loop essential for the proper function of the enzyme. Group X sPLA$_2$ (sPLA$_2$-X) exhibits the highest binding affinity of the sPLA$_2$s towards phosphatidylcholine, which is the major membrane phospholipid in eukaryotes, and plays important, but not fully understood, roles in physiological and pathophysiological processes in various mammalian tissues.[1]

Human sPLA$_2$-X is synthesized as a preproprotein that is processed to a 134 amino acid precursor protein (zymogen) whose N-terminal 11-residue propeptide is then cleaved off to result in the mature enzymatic form. It is N-glycosylated, and has 8 intramolecular disulphide bonds and the structural features of both group I and II sPLA$_2$s.[2–4] sPLA$_2$-X exhibits comparable abilities to hydrolyze zwitterionic and anionic interfaces and, when added exogenously to mammalian cells, is thus able to release free arachidonic acid (AA) from the intact plasma membranes and from low-density lipoproteins.[3,5,6]

sPLA$_2$-X expression has been detected in various human tissues, including spleen, thymus, pancreas, placenta, lung, heart, brain, testis, prostate, colon, and small intestine.[7–9] Recent studies suggested distinct roles of sPLA$_2$-X in various cellular processes and tissues. These include activation of certain inflammatory cells,[10] such as macrophages[11] and mast cells,[12] thus contributing to inflammatory lung disease,[13] and roles in other autoimmune and allergic diseases. Inflammatory responses in airway epithelial cells suggest a role in lung injury[14] and asthma.[15] Release of lysophosphatidylcholine induce neurite outgrowth,[16] melanocyte pigmentation[17] and reduces corticosterone production by altering the expression of steroidogenic acute regulatory protein in the adrenal

glands.[18] Modification of low-density lipoprotein and release of AA suggest its role in pathophysiology of atherosclerosis.[19] Potency in the release of AA leading to cyclooxygenase-2-dependent prostaglandin E_2 formation suggest its role during colon tumorigenesis.[20] Many of the biological effects of $sPLA_2$-X have been attributed to its ability to generate bioactive lipid mediators, but it may also induce intracellular signalling events through processes independent of phospholipid hydrolysis, *i.e.* working as a ligand that binds with a high affinity to the M-type $sPLA_2$ receptor in the mammalian plasma membrane.[21]

As the biological roles of $sPLA_2$-X are not completely understood, its action on various cellular models is an important area of research. One of the approaches for such studies is also to exploit recombinant human $sPLA_2$-X. An effective procedure for overexpression and simple purification of human $sPLA_2$-X is therefore required. In addition, large amounts of recombinant enzyme are needed for the design of specific inhibitors to studying the activities of these enzymes.

Human $sPLA_2$-X has been expressed in recombinant form as a fusion protein with the N-terminal portion of glutathione *S*-transferase, with the yield of mature enzyme of about 1 mg per litre of bacterial culture.[3] A subsequent study reported an improved yield, but the expression and purification procedures still included a tryptic digestion of the fusion protein after the renaturation step.[22]

In this study, we report simple procedures for overexpressing mature human $sPLA_2$-X in the form of inclusion bodies in *Escherichia coli*, and for its refolding and purification to yield the catalytically active enzyme in milligram quantities.

2. Experimental

2. 1. Plasmid Construction

A full-length cDNA encoding human $sPLA_2$-X (gift of Dr. Petan) was used as a template for a PCR performed with two oligonucleotides, N2-hGX (5'-CGAATTC**CAT ATG** GGA ATA CTG GAA CTG GCA GGA ACT GTG GGT TG-3'), corresponding to the N-terminal end of mature $sPLA_2$-X, carrying an *Nde*I site (bold) and two silent mutations (underlined), and C-hGX (5'- CGAATTC**AAG CTT**CAG TCA CAC TTG GGC GAG TCC GGC TCA CA-3'), corresponding to the C-terminal end of $sPLA_2$-X with a stop codon, carrying a *Hin*dIII site (boldfaced). The PCR reaction mixture contained (in 50 µl total volume): 5 µl of 10x *Pfx* amplification buffer (Invitrogen), 1 µl of 50 mM $MgSO_4$, 6 µl dNTP mix (0.3 mM final concentration), 100 pmol of each primer, approximately 20 ng target cDNA and 1 U of Platinum® *Pfx* DNA polymerase (Invitrogen). The following cycles were performed: 2 min at 95 °C; 25 cycles, each composed of 45 s denaturation at 94 °C, 45 s of annealing at 53.5 °C, and 1 min of extension at 70 °C; 2 min at 72 °C and finally held at 4 °C. The PCR-amplified fragment, coding for mature human s-PLA_2-X, was digested with *Nde*I and *Hin*dIII endonucleases and inserted into the pJP4.1 expression vector (Figure 1), used previously for bacterial production of a snake group IIA $sPLA_2$, ammodytoxin A (AtxA).[23] The PCR product was confirmed by nucleotide sequencing.

2. 2. Recombinant Expression of Human $sPLA_2$-X in *Escherichia coli*

Mature human $sPLA_2$-X was expressed in *Escherichia coli* BL21(DE3) host cells grown in 1 litre of LB-M9 medium containing ampicillin (100 µg/ml). Cells were grown to an OD_{600} ~1.5, then induced with isopropyl-1-thio-β-D-galactopyranoside (1 mM) for 3 h at 37 °C. Cells were pelleted and resuspended in 100 ml of TES buffer (50 mM Tris-HCl, pH 8.0, 40 mM EDTA, 25% (m/v) sucrose) on ice. The following reagents were added (final concentrations): lysozyme (1 mg/ml), DNase (10 µg/ml), RNase (20 µg/ml), and 0.1% (v/v) Triton X-100. The suspension was homogenized with an Ultra Turrax homogeniser (Janke & Kunkel, IKA-Labortechnik, Germany), 3 times for 30 s, incubated on ice for an hour and occasionally shaken vigorously. Inclusion bodies were collected by centrifugation at 4,500 rpm (GS-3, Sorvall, USA) for 40 min and washed with 0.5 M urea in TE buffer (50 mM Tris-HCl, pH 8.0, 40 mM EDTA), 1 M urea in TE buffer and twice with TE buffer. The inclusion body pellet was stored at –80 °C until use.

2. 3. Solubilisation of Inclusion Bodies and Refolding

The resulting pellet was solubilised in 100 ml of 6 M guanidine-HCl, 0.3 M Na_2SO_3, pH 8.3, and proteins were fully S-sulphonated by adding 0.05 volume of Thannhauser reagent[24] for 1 h at room temperature. The reaction was stopped by adding 1% (v/v, final) acetic acid and left overnight at 4 °C. The precipitated sulphonated protein was collected by centrifugation at 5,000 rpm (GS-3) for 15 min at 4 °C. After this step, two separated refolding methods were evaluated using either refolding buffer previously used for glutation-*S*-transferase fusion of recombinant $sPLA_2$-X or for recombinant AtxA. In the first method, the protein pellet was dissolved at 10 mg/ml in 5 M guanidine-HCl, 50 mM Tris-HCl, pH 8.0, and added dropwise to 1 litre of refolding buffer (0.9 M guanidine-HCl, 50 mM Tris-HCl, pH 8.0, 0.8 M NaCl, 10 mM $CaCl_2$, and 5 mM cysteine) with constant stirring at room temperature. Stirring was continued for 10 min, then the solution was allowed to stand without stirring at room temperature for 2–3 days. In the second method, the protein pellet was dissolved at 10 mg/ml in 5 M guanidine-HCl, and added dropwise to 1 litre of refolding buffer for AtxA (1 M guanidine-HCl, 25 mM boric acid (pH 8.0), 10 mM $CaCl_2$, 8 mM cysteine, 1 mM cystine, 1 mM EDTA) and the solu-

tion allowed to stand 2–3 days without stirring at 4 °C. Enzymatic PLA_2 activity was monitored with a fluorometric assay (see below) until the activity stopped increasing. The refolded protein was concentrated by ultrafiltration to 50 ml with YM-10 membrane (Pall Life Sciences, USA) at 4 °C and dialyzed against pre-chilled buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM $CaCl_2$). Finally, the protein solution was concentrated to 30 ml, filtered through a 0.22 μm pore size membrane (Vacuum filtration system, TPP, Switzerland) and stored at 4 °C until purification.

2. 4. Purification of $sPLA_2$-X

Protein solution was loaded on a C4 reverse-phase HPLC column equilibrated with solvent A (0.1% trifluoroacetic acid). $sPLA_2$-X was eluted at 1 ml/min using water/acetonitrile with 0.1% trifluoroacetic acid (0% acetonitrile over 3 min, followed by 0–50% acetonitrile over 25 min, then by 50–100% acetonitrile over 3 min). Recombinant human $sPLA_2$-X eluted at ~19 min. It was concentrated on a vacuum concentrator (Savant, USA) and dissolved in MilliQ water. The purity of the protein was assessed by sodium dodecylsulphate-polyacrylamide gel electrophores (SDS-PAGE).

2. 5. Analytical Methods

Electrospray ionization mass spectrometry (ESI-MS) analysis of $sPLA_2$-X was performed using a Q-Tof Premier mass spectrometer (Waters, U.K.). The N-terminal sequence was determined by an Applied Biosystems Procise 492A protein sequencing system. SDS-PAGE was performed on a Mini Protean III electrophoresis apparatus (Bio-Rad, USA) in the presence of 150 mM dithiothreitol on 15% (w/v) polyacrylamide gels, with Coomassie Brilliant Blue R250 staining.

Secondary structure of mRNA was calculated and predicted using the RNAstructure program, Version 4.6, based on a dynamic programming algorithm.[25] A stretch of the first 110 nt of human $sPLA_2$-X mRNA transcribed from the bacterial expression vector was analysed for the presence of potential secondary structures.

2. 6. Phospholipase A_2 Hydrolytic Activity Assay

The enzymatic activity of $sPLA_2$-X was determined using a sensitive fluorometric $sPLA_2$ assay with small unilamellar phospholipids vesicles composed of 1-palmitoyl-2-pyrenedecanoyl-*sn*-glycero-3-phosphoglycerol (Life Science, USA) on a Safire2 microplate fluorescence detection system (Tecan, Switzerland).[26] The initial rate of hydrolysis of phospholipid vesicles by $sPLA_2$-X was carried out with a fatty acid-binding protein (FABP) assay using 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) as substrate essentially as described previously.[27]

3. Results and Discussion

Mature human $sPLA_2$-X has a Gly residue at the N-terminus, which is suitable for its direct heterologous expression, *i.e.* as a non-fused protein, in a bacterial cell. Namely, it has been shown that the initial Met residue is efficiently removed in proteins synthesized in *E. coli* if it is followed by a residue with a small side chain, such as Gly or Ala.[28] Our rationale was therefore to obtain non-fused human $sPLA_2$-X in the bacterial cytoplasm in the form of insoluble and inactive inclusion bodies that would be easily isolated, and then solubilised and refolded to a native conformation with all the 8 intramolecular Cys–Cys bonds properly formed. However, in contrast to previous reports on the bacterial expression of human $sPLA_2$-X fused to the N-terminal part of glutathione *S*-transferase,[3,22] the subsequent proteolytic step, which is time-consuming and difficult to control, could be omitted.

To provide effective production of $sPLA_2$-X during its direct heterologous expression in *E. coli*, we used a T7 RNA polymerase promoter-based vector and introduced

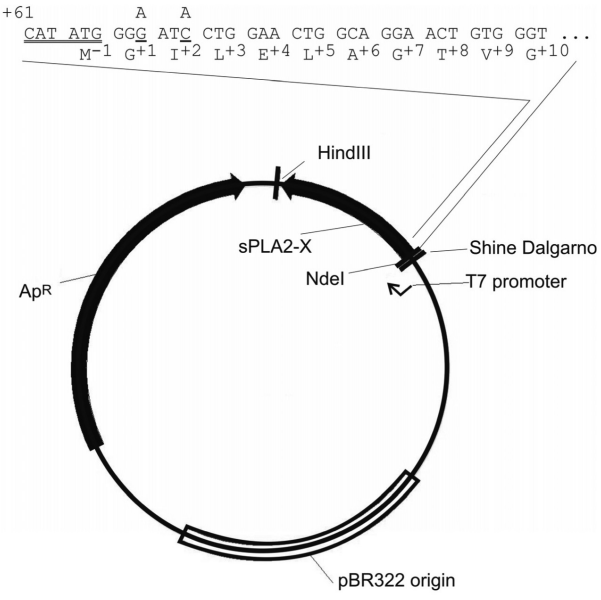

Figure 1: A schematic representation of the $sPLA_2$-X expression vector. The thick arrows denote the coding sequence of human $sPLA_2$-X and ampicilin resistance gene encoding β-lactamase (Ap^R). pBR322 origin, origin of replication; *Nde*I and *Hin*dIII, recognition sites for endonucleases *Nde*I and *Hin*dIII used for construction; a Shine-Dalgarno sequence (AGGAGA) is present at nucleotide positions 51–56 of the mRNA. The *Nde*I recognition site (doubly underlined) and two silent mutations introduced at nucleotide positions 69 and 72 (underlined) of the mRNA transcribed from the T7 promoter are shown, and the corresponding N-terminal amino acid sequence of human $sPLA_2$-X.

silent mutations into the initial codons of its cDNA. The mRNA secondary structure in the translation initiation region is one of the most important factor in the determining the efficacy of translation initiation in bacteria.[29,30] Therefore, as with the previous successful bacterial expression of snake group IIA $sPLA_2$s,[31] we introduced two silent mutations in the first two codons GGG (Gly1 of human $sPLA_2$-X) and ATC (Ile) to result in the two synonymous codons GGA and ATA, respectively (Figure 1). These mutations, first, eliminate an internal *Bam*HI (GGATCC) restriction site and, second, more importantly, also largely prevent the formation of a relatively stable hairpin structure presented in the predicted secondary structure of its mRNA and thus reduces the possibility of low protein level in the bacterial cell.

After 3 h induction with IPTG, transformed bacteria exhibited a high level of 14 kDa protein band, which was detected in whole-cell extracts on a SDS-PAGE gel (Figure 2). As expected, the $sPLA_2$-X is expressed in the form of insoluble inclusion bodies, which were solubilised and washed. The protein was then S-sulphonated in a two-step process and the solubilised protein precipitate renatured in a refolding buffer, similar to those used for glutathione *S*-transferase fusion of recombinant $sPLA_2$-X or for the production of recombinant AtxA (see above in the Experimental section).[3,22,23] The highest phospholipase A_2 activity was observed after 3 days in a refolding buffer similar to that used for glutathione *S*-transferase fusion of recombinant $sPLA_2$-X. The refolding was not accompanied by a significant amount of protein precipitation, which could in principle improve the refolding yield. A larger amount of protein precipitation and lower phospholipase A_2 activity were observed using the refolding conditions used for production of recombinant AtxA, and the isolation of recombinant protein in that case was therefore not pursued further.

After refolding, the recombinant $sPLA_2$-X was concentrated and dialysed in pre-chilled buffer to remove the remaining guanidine-HCl. During dialysis and concentration minor precipitation occurred, which was removed by filtration. The protein was purified by RP-HPLC, with three major components being separated. The first peak (F1 in Figure 3) contained phospholipase A_2 activity, and SDS-PAGE analysis of this fraction demonstrated that the single step RP-HPLC purification method resulted in a 14 kDa recombinant protein, with purity greater than 95%. The N-terminal amino acid sequencing (Gly1-Ile2-Leu3-...) verified the identity and purity of $sPLA_2$-X. No additional Met due to direct bacterial expression was present preceding the first (Gly1) residue of mature recombinant human enzyme in the first peak. The second peak represented the Met-form of recombinant human $sPLA_2$-X (Met(-1)-Gly1-Ile2-Leu3-...) and the third peak chicken egg white lysozyme (Lys1-Val2-Phe3-...), added to lyse the bacterial cells during recombinant protein isolation, both relatively well separated from the correctly *in vivo* processed and active mature enzyme in the first peak. Relative molecular mass determined by electrospray ionization mass spectrometry (ESI-MS) analysis of $sPLA_2$-X was 13,613.0, fairly close to the calculated value of 13,615.5 (Figure 4). The overall yield of purified recombinant $sPLA_2$-X (*i.e.* of the first RP-HPLC peak) was approximately 10 mg of protein/litre of culture. The increased yield in comparison to previous report[3] probably results from effective production of the enzyme, better renaturation of a non-fused protein (also reflected by low protein precipitation after refolding) and elimination of the protein cleavage step.

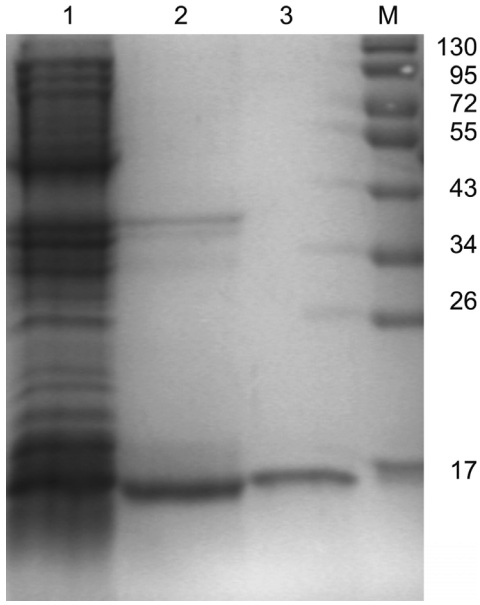

Figure 2: SDS-PAGE analysis of bacterial expression and purification of human $sPLA_2$-X. Lane M, protein molecular mass standards; lane 1, total proteins from IPTG-induced *E. coli* BL21(DE3) cells containing expression plasmid; lane 2, isolated inclusion bodies; and line 3, purified $sPLA_2$-X.

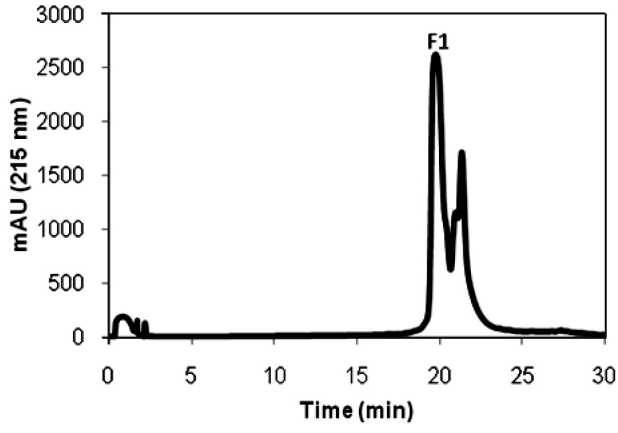

Figure 3: HPLC purification of refolded recombinant human $sPLA_2$-X. RP-HPLC was carried out using an HP 1100 Analyser instrument (Hewlett Packard, USA), connected to a C4 column. Pure human recombinant $sPLA_2$-X eluted at ~19 min (F1, first peak).

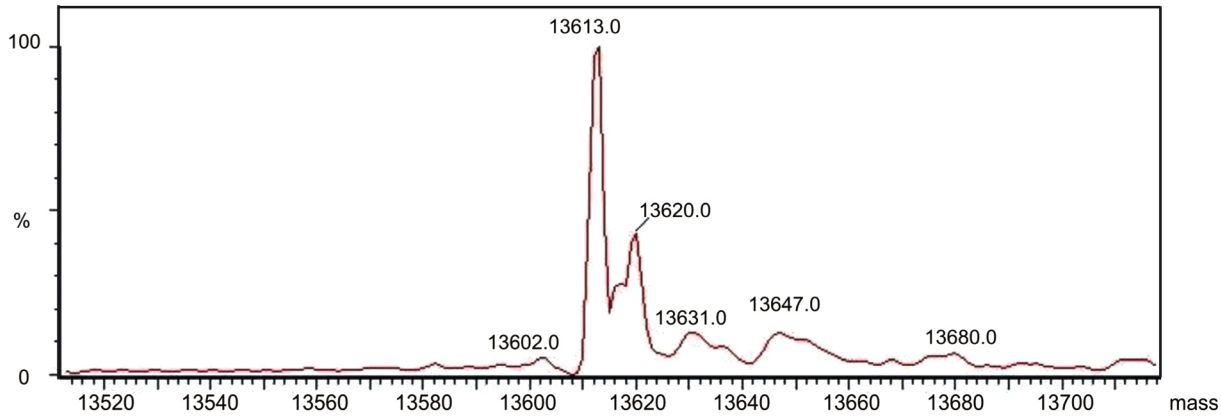

Figure 4: ESI-MS analysis of purified $sPLA_2$-X. Electrospray ionization mass spectrometry analysis was performed using a Q-Tof Premier mass spectrometer (Waters, U.K.). The protein samples were introduced into an electrospray nebulizer at a flow rate of 10 μl/min with a syringe pump. The spectra were obtained by scanning from *m/z* 2500 to 200 at 10 s/scan. Calibration was performed by sodium iodide cluster ions.

Specific enzymatic activity of $sPLA_2$-X derived from the initial velocity was determined on large unilamellar vesicles (0.1 μm in diameter) composed of single phospholipid substrates, either POPG or POPC, using real time fluorometric assay employing fatty acid-binding protein. $sPLA_2$-X shows high activity on POPG or POPC vesicles and, as expected, no lag phase was observed (Figure 5). Our determined specific activities (average ± SD) by the recombinant $sPLA_2$ were 47 ± 19 μmol min^{-1} mg^{-1} on POPG substrate and 7 ± 3 μmol min^{-1} mg^{-1} on POPC substrate, which is in the range of that previously determined for human and mouse $sPLA_2$-X.[3,5,32]

The recombinant human $sPLA_2$-X that we effectively produced in *E. coli* is practically identical to the natural mature enzyme. The only difference is that the recombinant protein is not N-glycosylated, while the natural counterpart is glycosylated at Asn71. However, it has been demonstrated that the N-glycosylation of human $sPLA_2$-X is not essential for its enzymatic as well as substrate specificity.[32] Results from neuronal cells transfected with human $sPLA_2$-X suggest that the zymogen is processed before or after secretion depending on cell types, and that the N-glycosylated form of $sPLA_2$-X may facilitate its secretion.[16] Interestingly, in contrast to the human orthologous enzyme, mouse $sPLA_2$-X is apparently not N-glycosylated.[33]

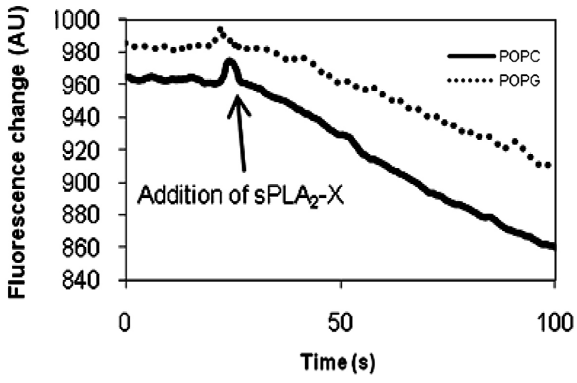

Figure 5: Initial rate of hydrolysis of POPG and POPC vesicles by recombinant human $sPLA_2$-X. All assays was carried out in Hank's balanced salt solution with 1.27 mM Ca^{2+} and 0.9 mM Mg^{2+}. Assays contained 10 μM of fatty acid-binding protein, 1 μM of 11-dansylundecanoic acid (Molecular Probes Inc.), and 30 μM phospholipid (POPG or POPC) extruded vesicles. The final assay volume was 1.3 ml, present in a fluorescence cuvette with a magnetic stir bar at 25 °C. Excitation was at 350 nm and emission at 500 nm with both slits at 10 nm. Assays were calibrated by adding a known amount of oleic acid and measuring decrease in fluorescence. Reactions were started by adding 10 ng of $sPLA_2$-X to POPG and 70 ng to POPC vesicles.

4. Conclusions

We report a successful bacterial expression, refolding and simple purification of catalytically active human $sPLA_2$-X. In comparison to previous reports, the final yield of purified recombinant enzyme has been significantly increased up to 10 mg per litre of bacterial culture. This is largely achieved due to the high expression and effective *in vitro* refolding of mature, non-fused enzyme. The purified recombinant human $sPLA_2$-X with the specific enzymatic activities comparable to those of natural protein pave the way for designing new PLA_2-X specific inhibitors, and provide a valuable tool for studying the cellular roles and functions of this effective membrane-active enzyme.

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Povzetek

Sekretorna fosfolipaza A_2 skupine X (sPLA_2-X) je eden od najbolj učinkovitih sesalskih PLA_2-encimov pri hidrolizi plazemskih lipoproteinov in fosfolipidov v membranah intaktnih celic, predvsem zaradi svoje relativno visoke afinitete vezave na elektro nevtralne fosfolipidne substrate, kot je npr. fosfatidilholin. Produkti njenega encimskega delovanja, lizofosfolipidi in proste maščobne kisline, še zlasti arahidonska kislina, so vpleteni v različne fiziološke in patološke procese, ki jih v zadnjem času poglobljeno raziskujejo. Kljub številnim raziskavam biološke vloge sPLA_2-X še niso povsem razjasnjene. Z namenom, da bi izvedli različne celične študije in načrtovali učinkovite encimske inhibitorje, smo pripravili večjo količino rekombinantne človeške sPLA_2-X. V članku opisujemo učinkovit ekspresijski sistem bakterije *Escherichia coli*, kot tudi *in vitro* renaturacijo in enostaven postopek čiščenja, ki omogoča donos do 10 mg človeške s-PLA_2-X na liter kulture. Za razliko od naravnega proteina je bil rekombinantni encim pridobljen v bakterijskih celicah brez N-terminalnega propeptida, tj. kot zrel protein, in ni bil N-glikoziliran, je pa zadržal vse encimske lastnosti pri hidrolizi vezikularnih substratov s fosfatidilglicerolom ali fosfatidilholinom.