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Recovery of functionally active recombinant human phospholipid scramblase 1 from inclusion bodies using *N*-lauroyl sarcosine

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Abstract Human phospholipid scramblase (hPLSCR1) is a transmembrane protein involved in rapid bidirectional scrambling of phospholipids across the plasma membrane in response to elevated intracellular calcium (Ca^{2+}) levels. Overexpression of recombinant hPLSCR1 in *Escherichia coli* BL21 (DE3) leads to its deposition in inclusion bodies (IBs). *N*-lauroyl sarcosine was used to solubilize IBs and to recover functionally active hPLSCR1 from them. Protein was purified to homogeneity by nickel-nitrilotriacetic acid (Ni^{2+}–NTA) affinity chromatography and was >98% pure. Functional activity of the purified protein was validated by in vitro reconstitution studies, ~18% of 7-nitrobenz-2-oxa-1, 3-diazol-4-yl-phosphatidylcholine (NBD-PC) phospholipids was translocated across the lipid bilayer in the presence of Ca^{2+} ions. Far ultraviolet circular dichroism (UV-CD) studies reveal that the secondary structure of protein is predominantly an α-helix, and under nondenaturing conditions, the protein exists as a monomer. Here we describe a method to purify recombinant membrane protein with higher yield than previously described methods involving renaturation techniques.

Keywords Human phospholipid scramblase 1 · Overexpression · Inclusion body · Tryptophan fluorescence · EF-hand motif · *N*-lauroyl sarcosine

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Introduction

The human phospholipid scramblase family (hPLSCR) consists of four homologous members named hPLSCR1–4, which are conserved from *Caenorhabditis elegans* to humans. Isoforms hPLSCR2–4 share approximately 59%, 47%, and 46% identity to hPLSCR1, respectively which is a 37-kDa type 2, single-pass membrane protein belonging to the adenosine triphosphate (ATP)-independent class of phospholipid translocators [1]. hPLSCR1 mediates bidirectional, nonspecific scrambling of phospholipids across the lipid bilayer in response to increased cytosolic calcium (Ca^{2+}) levels. It possesses a single EF-hand-like Ca^{2+} binding motif [^{273}DADNFGIQFPLD^{284}], and mutations in these residues resulted in loss of scramblase activity [25]. Ca^{2+} binding is associated with conformation change of hPLSCR1 and increased protein light scattering [18]. hPLSCR1 is also known to localize to the nucleus, and its shuttling to plasma membrane and nucleus is regulated by palmitoylation of cysteine rich motif [^{184}CCCPCC^{189}] [22]. Studies have shown that hPLSCR1 is activated in cellular processes such as apoptosis, cellular signalling, and cell coagulation during which a 1,000-fold increase in intracellular Ca^{2+} levels is observed [4, 15, 23]. Homology modeling studies reveal that hPLSCR1 is closely related to At5g01750 of the DUF567 family. Based on the model, it is proposed that hPLSCR1 forms a closed symmetric β-barrel comprising 12 β-strands wrapped around a hydrophobic C-terminal helix [2]. However, very little is known about the functioning of hPLSCR1.

Recombinant hPLSCR1 is obtained by cloning and overexpression in *Escherichia coli*; however overexpression leads to its deposition in inclusion bodies (IBs). Traditional techniques of purification are limited by protein aggregation and low recovery yields. It was reported that

urea-assisted refolding and purification of hPLSCR1 was achieved with <10% recovery from IBs [16]. Alternatively, the solubility of hPLSCR1 was increased by coexpressing the protein with chaperones as well as a fusion with maltose binding protein (MBP). However, limitations with these methods include stabilization of protein after tag removal and the additional purification steps required to obtain the pure protein [17, 26].

IBs have long been considered as aggregates of misfolded inactive proteins. Interestingly, recent studies show that IBs have a considerable proportion of correctly folded protein, which can be extracted without applying any renaturation steps. Enzymatic activity in IBs was reported several years ago, and since then, many studies have shown the existence of functionally active proteins inside the IBs of G-CSF, GFP, His_7-dN6TNF-α, ΔN19LT-α, and actin proteins [14, 19, 24]. *N*-lauroyl sarcosine (0.2–3%) has been effectively used to extract correctly folded proteins from IBs. Based on this strategy, we developed a simple and efficient procedure to purify functionally active recombinant membrane protein (hPLSCR1) from IBs. This procedure requires single-step affinity purification with yields up to 5.5–6.5 mg g^{-1} cell mass. This strategy can also be applied for other membranous and hydrophobic proteins, which aggregate as IBs in bacterial expression systems.

Materials and methods

Materials

N-lauroyl sarcosine (NLS), egg phosphatidylcholine (egg PC), and phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma (USA), *E. coli* DH5α and *E. coli* BL21(DE3) strains were obtained from ATCC. Complementary DNA (cDNA) of hPLSCR1 was purchased from Invitrogen (USA) and pET-28 b(+) from Novagen (USA). Fluorescent labeled lipid, 7-nitrobenz-2-oxa-1, 3-diazol-4-yl-phosphatidylcholine (NBD-PC) was purchased from Avanti Polar Lipids, Inc. (USA). SM2 Bio-Beads, Chelex-100 resin and protein molecular weight markers were obtained from Biorad (USA). The nickel-nitrilotriacetic acid (Ni^{2+}-NTA) matrix was purchased from Qiagen (USA). Isopropyl β-D-1-thiogalactopyranoside (IPTG), dithiothreitol (DTT), ethylene glycol tetraacetic acid (EGTA), molecular biology grade calcium chloride ($CaCl_2$), and other routine chemicals were purchased from Himedia (India).

Construction of expression vector

The open reading frame (954 base pairs) of hPLSCR1 was amplified from the mammalian expression vector pCMV-SPORT6 by polymerase chain reaction (PCR) using forward primer 5′-ATATCATATGATGGATAACAGAAC-3′ and reverse primer 5′- ATTATGCGGCCGCCCACACGC CGCTTTTC-3′ containing the *Nde*1 and *Not*1 sites, respectively. The amplicon was digested with the *Nde*I and *Not*I, and the fragment was ligated to the *Nde*I and *Not*1 sites of pET-28 b(+) expression vector. The cloned insert was further confirmed by nucleotide sequencing.

Overexpression of hPLSCR1

E. coli BL21 (DE3) cells containing *hPLSCR1* in pET-28 b(+) was inoculated into a selective Luria Bertani broth (LB) containing kanamycin (50 mg l^{-1}). Growth was carried out at 37°C, at 180 rpm till the optical density measured at a wavelength of 600 ($O.D_{600}$) reached ~0.5, and cells were then induced with 0.1 mM IPTG and induction carried out for another 4 h. Cells were then harvested by centrifugation for 5 min at 10,500×*g* and 4°C. Induced cells (1 g) were resuspended in 10 ml buffer containing 20 mM Tris–hydrochloride (HCl), pH 7.5, 200 mM sodium chloride (NaCl), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetate (EDTA), and 1 mM dithiothreitol (DTT). Cell suspension was subjected to probe sonication for 2 min (1 s on/2 s off) at 30% amplitude. The soluble and insoluble fractions were separated by centrifugation at 4°C for 20 min at 18,000×*g*, and localization of the overexpressed protein was analyzed on 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). Target protein was estimated by densitometric analysis using Quantity One software (Bio-Rad).

Solubilization and purification of hPLSCR1

The insoluble fraction was resuspended in 10 ml buffer A (20 mM Tris–HCl, 200 mM NaCl- pH 7.5) with varying NLS concentrations from 0.1% to 2% (w/v). The suspension was agitated at 130 rpm for 24 h at 20°C, followed by centrifugation at 4,400×*g* for 15 min at 4°C. Subsequently, NLS was replaced with 0.025% (w/v) Brij-35 (buffer B) in all purification steps. The supernatant was then subjected to step dialysis with 20-kDa cut-off membrane against buffer B, maintaining the protein concentration at 100 μg/ml, with three buffer changes after every 12 h. Absorbance at 215 nm was used to determine the amount of residual NLS present after dialysis. The protein solution was then filtered through a 0.22-μm membrane filter and loaded onto an Ni^{2+}–NTA column, equilibrated with the buffer B prior to use. Binding was carried out for 10 min at 25°C, and the protein was finally eluted out with buffer B containing 250 mM imidazole. The eluted protein was then repeatedly dialyzed against buffer B made from water treated with

Chelex-100 resin (Bio-Rad) to completely remove the imidazole and also make the protein free from metal ions.

Size-exclusion chromatography

The molecular weight of purified hPLSCR1 was determined by size-exclusion fast protein liquid chromatography (FPLC) using Superdex-200 column. The elution was carried out using buffer B with a flow rate of 1 ml/min. Protein standards were used to calibrate the column; total column volume was 120 ml, with a void volume (Vo) of 35.19 ml. From the standard plot, the approximate molecular weight of the purified protein was determined using the elution volumes (Ve).

Reconstitution of hPLSCR1 into proteoliposomes

Symmetrically labeled vesicles were made as described previously [8]. Briefly, 4.5 μmol of egg PC and 0.3 mol% of NBD-PC was mixed and dried under nitrogen to completely remove the chloroform. The lipids were then solubilized in reconstitution buffer containing 10 mM hydroxyethyl-1-piperazine ethanesulfonic acid/sodium hydroxide (HEPES/NaOH) (pH 7.5), 200 mM NaCl, and 1% (w/v) Triton X-100, along with 100 μg of purified protein. Experiments wherein hPLSCR1 was not affinity-purified, NLS solubilized IBs were directly used for reconstitution. Detergent was slowly removed by addition by SM2 Bio-Beads (0.1 g h^{-1}). Proteoliposomes were collected by centrifugation at 2,30,000×*g* in an MLA130 rotor (Beckman Coulter ultracentrifuge, USA) for 45 min at 4°C. The vesicles were then extruded through a lipid extruder (Avanti Inc) using 0.1-μm polycarbonate membrane filter to make unilamellar symmetrically labeled vesicles. Asymmetric vesicles were then prepared by treating the symmetrically labeled vesicles with 20 mM dithionite for 20 min, followed by extensive washing to remove traces of dithionite.

Scramblase assay

Scramblase activity was measured for the inside-labeled proteoliposomes, as described previously [16] (see electronic supplementary material figure for assay scheme). Briefly, proteoliposomes containing NBD-PC were incubated for 90 min at 37°C in 10 mM HEPES/NaOH, pH 7.5, 100 mM NaCl in the presence of 2 mM Ca^{2+} or 4 mM EGTA and was transferred to a stirred spectrofluorometer (Perkin Elmer LS-55). Sample fluorescence (excitation 470 nm and emission 532 nm) was monitored continuously at 25°C with constant low-speed stirring and slit widths of 3 nm (excitation) and 5 nm (emission). The initial fluorescence was recorded in a spectrofluorometer for 100 s, 20 mM dithionite was added, and the fluorescence was monitored for the next 400 s. The difference between the nonquenchable fluorescence observed in the presence and absence of Ca^{2+} is attributed to the Ca^{2+}-induced scrambling of NBD-PC located in the inner leaflet of proteoliposomes.

Intrinsic tryptophan fluorescence

Intrinsic fluorescence of protein was measured using Perkin Elmer LS-55 fluorescence spectrofluorometer. All plastic wares and cuvettes were soaked in 10 mM EDTA, rinsed with ion-free distilled water (Millipore, Bedford, PA, USA), and dried before use. Protein (1.5 μM) was used for steady-state fluorescence experiments. Spectra were recorded by exciting the sample at 280 nm with excitation and emission slit widths set at 8 nm and 10 nm, respectively. Emission spectra were recorded from 300 nm to 500 nm at 25°C, with a scanning speed of 100 nm min^{-1}. For data evaluation, buffer blanks were subtracted from the spectra. Intrinsic tryptophan fluorescence measurements for denatured hPLSCR1 were carried out by incubating the protein with 6 M guanidine hydrochloride (GnHCl) at 25°C overnight.

Circular dichroism

Circular dichroism (CD) spectra were obtained using JASCO J-810 spectropolarimeter (Easton, MD, USA) at 25°C using 0.1 cm path-length quartz cells. Spectra were acquired at 10 nm min^{-1}, time constant 0.5 s, 2-nm bandwidth, and the recorded spectra are an average of three scans. Protein (10 μM) was used for far ultraviolet (UV) CD. Background (buffer blank) spectra were collected under similar conditions and subtracted to obtain the final spectra.

Results

Overexpression of hPLSCR1

High-level expression of 6X His tag (His_6)-hPLSCR1 was achieved using pET-28 b(+) vector in *E. coli* BL21 (DE3) strain using IPTG as an inducer. In the absence of inducer, hPLSCR1 was not expressed in either soluble or insoluble fractions (lanes 1 and 4, Fig. 1). Following induction with IPTG, a prominent band appears in cell fraction (lane 2, Fig. 1) with less target protein in the soluble fraction (lane 5, Fig. 1). Densitometric analysis reveals that the overexpressed protein (lane 2, Fig. 1) is approximately 75% pure. The apparent molecular weight of the overexpressed hPLSCR1 was approximately 37 kDa on 12% SDS-PAGE

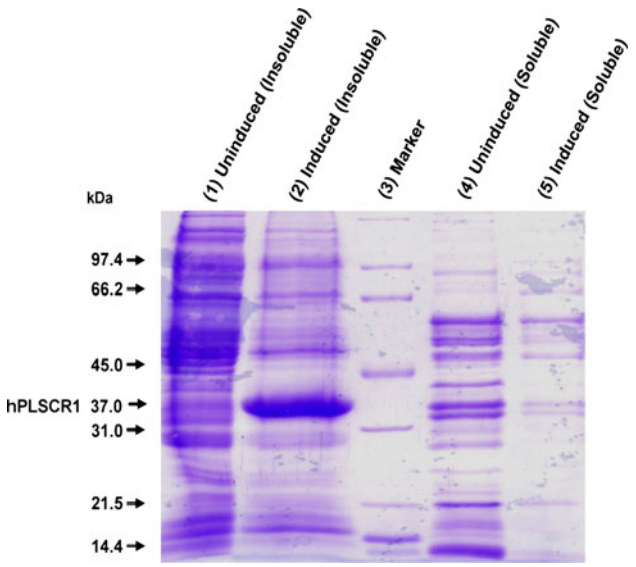

Fig. 1 Overexpression of 6X His tag (His_6)-human phospholipid scramblase-1 (hPLSCR1) in *Escherichia coli*. Cells were lysed and checked for protein expression on 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE)

(lane 2), which matched with the earlier reported molecular weight [16]. Various parameters, such as varying temperature, IPTG concentration, and post induction times, which are commonly used to enhance solubility of the protein, did not localize hPLSCR1 into soluble fraction (data not shown). Therefore, cells were grown at 37°C, and protein expression was carried out for 3 h after addition of 0.1 mM IPTG.

Solubilization and recovery of hPLSCR1 using NLS

Solubilization of the IBs is a critical step toward obtaining a maximal amount of the desired protein in soluble form without inducing any chemical or deleterious modifications to it. Purification from IBs has an additional advantage, as it contains relatively more of the target protein. When hPLSCR1 IBs were solubilized with varying NLS concentrations (0.1–2% w/v), hPLSCR1 solubility (ratio of soluble protein to total protein) increased with an increase in NLS concentration up to 1% (w/v) NLS (Fig. 2a). Extractability of hPLSCR1 into the soluble form increased with increasing concentrations of NLS, and with 2% NLS, approximately 50% of IBs was effectively solubilized (Fig. 2b, c). Although at concentrations >2% NLS the percentage of hPLSCR1 in soluble form increased, solubilization of hPLSCR1 IBs >2% NLS was not attempted, as maximal functional activity of the extracted protein was observed at approximately 1–2% of NLS, after which there was no further increase in scramblase activity. This suggests that only a certain proportion of correctly folded protein is present inside the IBs that can be extracted using NLS.

Ni^{2+}–NTA purification of hPLSCR1

NLS-solubilized hPLSCR1 was then repeatedly dialyzed against equilibration buffer, as the presence of NLS inhibits binding to Ni^{2+}-NTA matrix. Residual NLS concentration after dialysis was determined by measuring absorbance at 215 nm using reverse-phase high performance liquid chromatography (RP-HPLC) (data not shown). Approximately 80% of solubilized protein bound to Ni^{2+}-NTA matrix, and the bound protein was eluted with buffer A containing 250 mM imidazole and 0.025% (w/v) Brij-35 (Fig. 2d). Brij-35, a non-ionic detergent, was included to stabilize the membrane protein. This one-step purification resulted in highly pure hPLSCR1 >98%. During the process of solubilization and purification, the recovery yield of hPLSCR1 was ~40% from the target hPLSCR1 in IBs. The molecular weight of the purified hPLSCR1 was estimated to be approximately 37 kDa from SDS-PAGE. Protein recovered at every step of NLS solubilization and purification is summarized in Table 1.

Size-exclusion chromatography

To determine molecular size and oligomeric state of the purified protein, size-exclusion chromatography was carried out. Purified hPLSCR1 was present in the monomeric state (~37 kDa) based on Ve (Fig. 3). This result is in agreement with an earlier method of hPLSCR1 purification in which the protein exits as a monomer [16].

Biochemical activity of purified recombinant hPLSCR1

Reconstitution of integral membrane proteins into phospholipid vesicles to form functional proteoliposomes is used to identify and characterize the mechanism of action of membrane proteins. To further confirm biochemical activity of the purified hPLSCR1 solubilized using various NLS concentrations, scramblase assay was performed. Inside-labeled vesicles incubated with EGTA (control) showed no change in fluorescence intensity upon dithionite addition. In case of proteoliposomes incubated with Ca^{2+}, significant decrease in fluorescence intensity was seen upon dithionite addition, indicating Ca^{2+} induced translocation of phospholipids from the inner to outer leaflet, which are subsequently quenched by dithionite (Fig. 4a). Approximately 18% NBD-PC (maximum activity) was translocated across the lipid bilayer in the presence of Ca^{2+} ions when IBs were treated with 2% NLS (Fig. 4b). No further increase in scramblase activity was observed when IBs where solubilized with NLS concentrations >2% (w/v). Similar rates of phospholipid translocation were observed using hPLSCR1 purified as MBP-hPLSCR1 fusion [26]. These results show that the purified hPLSCR1 was functionally active.

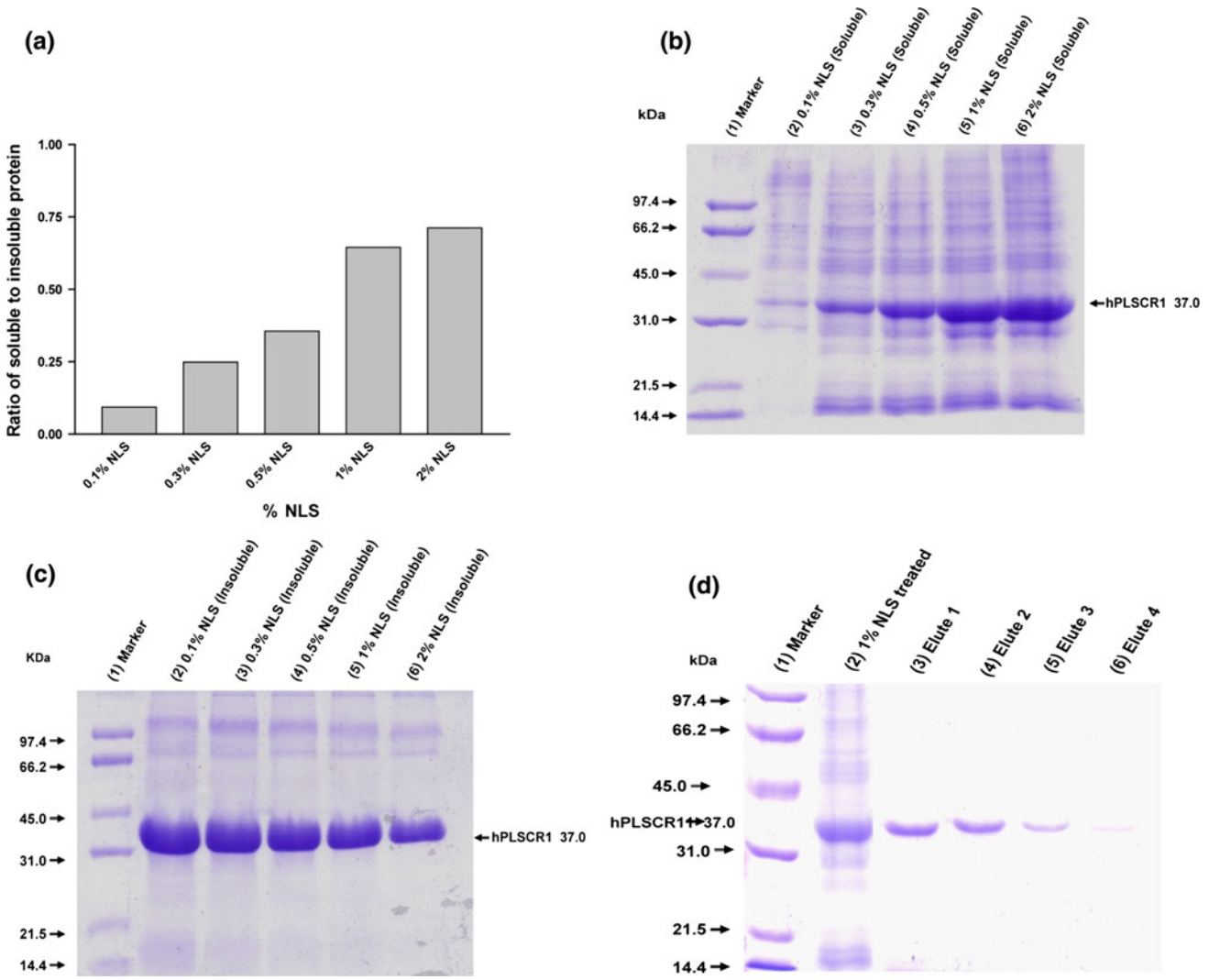

Fig. 2 Solubilization and purification of human phospholipid scramblase 1 (hPLSCR1) inclusion bodies (IBs) using varying concentration of *N*-lauroyl sarcosine (NLS). **a** Ratio of soluble protein to total protein of hPLSCR1 obtained from IBs solubilized with varying NLS concentration. **b** Soluble fractions of hPLSCR1 after NLS treatment. **c** Insoluble fractions after NLS treatment. **d** Purification of 1% NLS-treated hPLSCR1 using nickel nitrilotriacetic acid (Ni^{2+}-NTA) affinity chromatography

Table 1 Human phospholipid scramblase 1 (hPLSCR1) recovery from inclusion bodies (IBs) by *N*-lauroyl sarcosine (NLS) and its purification by nickel-nitrilotriacetic acid (Ni^{2+}–NTA) affinity chromatography

Stages of solubilization/purification	Total protein (mg)	Target protein (mg)	Purity (%)
Cell lysate (1 g cell wet weight in 10 ml lysis buffer)	35.6	19	53.3
Insoluble fraction	24.6	18	73.2
Soluble fraction (after 1% NLS treatment)	11.5	7.2	62.6
Purified hPLSCR1 (after Ni^{2+}–NTA purification)	6.5	6.5	98

Biochemical activity of recombinant hPLSCR1 without purification

In order to confirm the presence of functionally active hPLSCR1 in IBs, NLS-solubilized IBs were directly reconstituted to form proteoliposomes. Here, both Triton X-100 and NLS were simultaneously removed using SM2 Bio-Beads to facilitate the formation of proteoliposomes. Scramblase assay for these inside-labeled vesicles showed greater NBD-PC translocation for Ca^{2+}-incubated proteoliposomes than for those incubated with EGTA, confirming the presence of functionally active hPLSCR1 (Fig. 4c, data

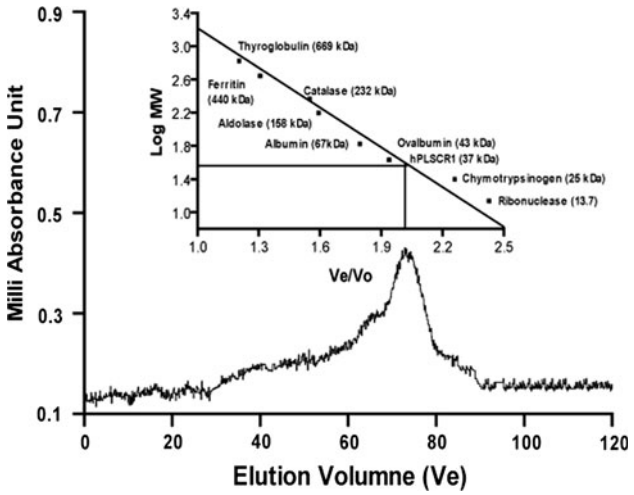

Fig. 3 Molecular size determination of purified human phospholipid scramblase 1 (hPLSCR1) by size-exclusion chromatography. Purified hPLSCR1 [elution volume/void volume (Ve/Vo = 2.06)] corresponds to the monomeric state of the protein (~37 kDa)

shown for 1% NLS-treated hPLSCR1). Similar experiments were carried out using various concentrations of NLS, and it was found that IBs when solubilized with 1% NLS showed highest scramblase activity (~14%) (Fig. 4d). Similar to purified hPLSCR1, although with increase in NLS concentration, more IBs were extracted into soluble form, maximum scramblase activity was achieved with 1–2% NLS. These results confirm the presence of functionally active hPLSCR1 inside IBs, which can be extracted using NLS.

Intrinsic tryptophan fluorescence of the recovered hPLSCR1

hPLSCR1 contains four tryptophan residues, and to assess whether the purified protein is in its native state, intrinsic tryptophan fluorescence was measured. Figure 5a shows that emission maxima spectra of purified hPLSCR1 was centered around 340 nm (blueshift) compared with denatured hPLSCR1 centered around 360 nm. Also, the intrinsic tryptophan fluorescence of GnHCl-denatured hPLSCR1 was significantly quenched compared with purified hPLSCR1 (Fig. 5a). This indicates that the tryptophan residues are folded into the inside of the purified protein.

Circular dichroism of the recovered hPLSCR1

In order to verify the secondary structure of the purified protein, far UV-CD spectra of the protein was measured and showed double negative minima at 209 and 222 nm (Fig. 5b), typical of a protein containing a large proportion of α-helical structure, and in good agreement with the published CD spectra of hPLSCR1-MBP fusion [18].

Discussion

The major problem with purifying membrane proteins is the inability to overexpress large quantities of functional protein. Moreover, crystallization studies of these membrane

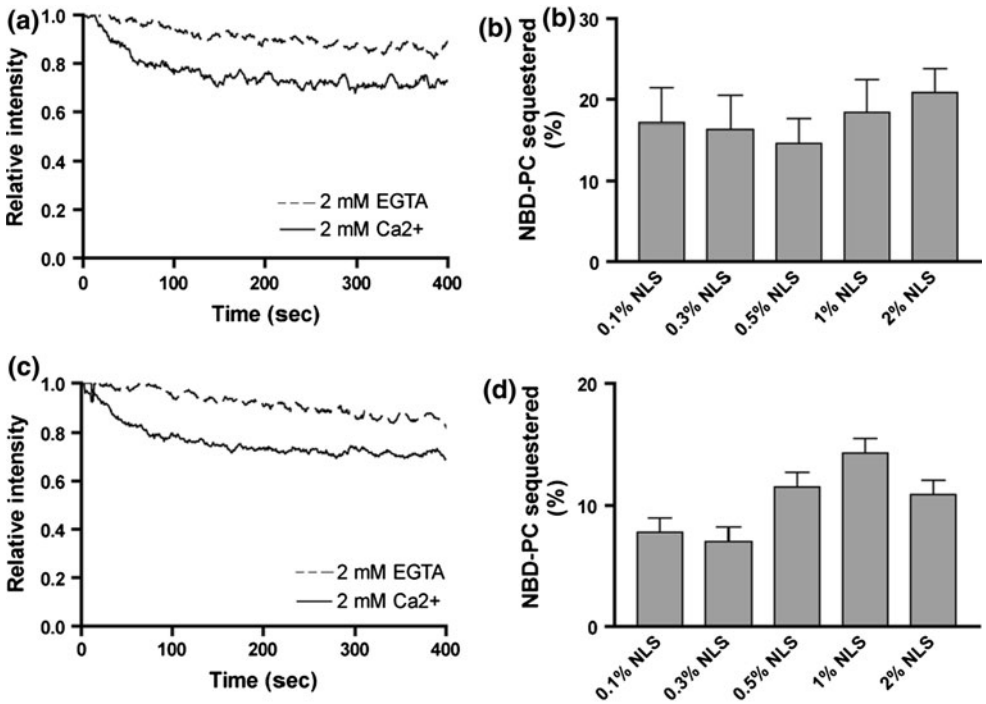

Fig. 4 Biochemical reconstitution and functional assay of human phospholipid scramblase-1 (hPLSCR1). **a** Scramblase activity of purified hPLSCR1. **b** Scramblase assay of purified hPLSCR1 obtained from inclusion bodies (IBs) solubilized with varying *N*-lauroyl sarcosine (NLS) concentrations. **c** Scramblase assay of hPLSCR1 after being solubilized with 1% NLS, reconstituted directly without nickel-nitrilotriacetic acid (Ni^{2+}-NTA) purification. **d** Scramblase of hPLSCR1 IBs solubilized with varying NLS concentration without Ni^{2+}-NTA purification

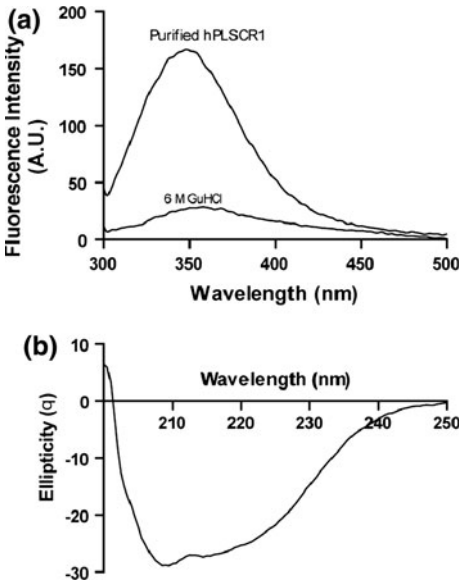

Fig. 5 Intrinsic tryptophan fluorescence and secondary structure of purified human phospholipid scramblase 1 (hPLSCR1). **a** Emission spectra of purified hPLSCR1 and guanidine hydrochloride (GnHCl) denatured hPLSCR1. **b** Far ultraviolet circular dichroism (UV-CD) of purified hPLSCR1

proteins require the purified protein to be stable and homogenous. Various parameters, such as detergent screening, detergent concentration, and buffer need to be optimized for the stability of membrane proteins. Heterologous overexpression of membrane proteins in *E. coli* generally leads to deposition of overexpressed protein as misfolded aggregates in inclusion bodies. General strategy for refolding membrane protein involves solubilization of IBs using denaturants such as GnHCl and urea. However, such refolding strategies are limited by hydrophobic interactions and incorrect disulfide bond formations, leading to protein aggregation and low recovery yields [7]. An alternate strategy for purifying proteins that form IBs involves overexpression as fusions with highly soluble proteins, such as glutathione-S-transferase, MBP, and thioredoxin [6, 11]. However, such strategies are limited by protein instability problems during fusion protein cleavage to remove the solubility tag.

High-level overexpression of hPLSCR1 was achieved using the expression vector pET-28 b(+) in *E. coli*. Protein was overexpressed as His_6-hPLSCR1 and mostly expressed in IBs. hPLSCR1 IBs were effectively solubilized using 8 M urea and purified to homogeneity using Ni^{2+}–NTA chromatography. However, this method was limited by low recovery yields <10% caused by protein aggregation and precipitation once the denaturant concentration was reduced. It is known that aggregate formation can be deterred by using additives; however, it is known that these additives do not work universally [9].

IBs have long been considered aggregates of misfolded proteins. However, this view is changing, as many reports indicate the presence of biological activity inside IBs. Infrared spectroscopy revealed the presence of correctly folded proteins inside the IBs of human granulocyte colony stimulating factor (hG-CSF) [10]. NLS, an ionic detergent, has been effectively used to solubilize IBs of various proteins and has an advantage of being removed easily by dialysis or ion exchange; also, it does not cloud at low temperatures.

NLS is known to stabilize membrane proteins by binding to hydrophobic patches, thereby preventing aggregation [12]. hPLSCR1 IBs were solubilized using various NLS concentrations ranging from 0.3–2% (w/v). Solubilized proteins were then affinity-purified using Ni^{2+}–NTA chromatography. However, in the presence of NLS, solubilized hPLSCR1 failed to bind to Ni^{2+}–NTA resin. This could be attributed to the masking of His_6 tag by detergent molecules, thereby preventing efficient binding. However, the presence of detergent is crucial for the stability of membrane proteins in order to mimic lipid environment [13]. Hence, NLS was replaced by nonionic detergent Brij-35, and binding to Ni^{2+}–NTA significantly improved. Overall recovery of protein purified by this method was approximately 98% pure, and the protein recovery yield was ~40% compared with ~10% by the urea refolding method. Using this method, approximately 5.5–6.5 mg of purified protein could be obtained per gram cell mass. Alternatively, we also show the presence of functionally active hPLSCR1 inside IBs by directly reconstituting NLS-solubilized IBs without purification. This study will further augment the existing reports on the presence of functional proteins inside IBs.

In order to determine whether the purified protein regained its approximate native conformation, CD spectra and tryptophan fluorescence spectroscopy was used [5, 21]. Far UV-CD of the purified protein reveals that the protein has an abundantly α-helix structure similar to native hPLSCR1. Tryptophan fluorescence reveals that emission spectra of purified hPLSCR1 and GnHCl denatured protein are distinct. In general, the more tryptophan residues buried inside the protein, the greater the blue shift in emission maximum of the protein [3, 20]. In the case of purified hPLSCR1, tryptophan residues are buried inside the hydrophobic core, whereas in the case of the GnHCl-denatured hPLSCR1, tryptophan residues are exposed on the surface, and as a result, the intrinsic tryptophan fluorescence of the protein is quenched. In order to further determine the oligomeric state of the purified proteins, size-exclusion chromatography was carried out. Based on the Ve/Vo, purified hPLSCR1 exists as a monomer, similar to earlier reported methods.

In summary, an efficient and rapid method for obtaining higher yields ($\sim$40%) of soluble hPLSCR1 from IBs using NLS is described. This purification strategy can also be applied to the purification of other membrane proteins from IBs without using any renaturation procedures.

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