Purification of Cytosolic Phospholipase A₂α C2-domain after Expression in Soluble Form in *Escherichia coli*

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[Abstract] Previous expression/purification strategies for cytosolic phospholipase A₂α C2-domain in *Escherichia coli* have relied on refolded protein recovered from inclusion bodies and sometimes containing C-terminal Cys139Ala and Cys141Ser substitutions to eliminate potential refolding complications induced by Cys residues. The protocol presented herein describes an effective method for the expression of cytosolic phospholipase A₂α C2-domain in soluble form in *E. coli* and subsequent purification to homogeneity. This protocol, which utilizes a cleavable 6xHis-SUMO tag, has recently been used to gain insights into the structural basis of phosphatidylcholine recognition by the C2-domain of cytosolic phospholipase A₂α (Hirano *et al.*, 2019)

Keywords: Cytosolic phospholipase A₂α, C2-domain purification, Soluble expression in *E. coli*, 6xHis-SUMO tag

[Background] Phospholipase A₂ (PLA₂) is a member of a diverse enzyme superfamily that hydrolyzes the *sn*-2 acyl ester linkage of phosphoglycerides (Smith, 1989; Dennis *et al.*, 2011; Mouchlis and Dennis, 2019). Cytosolic PLA₂α (cPLA₂α), a Group IV mammalian PLA₂ family member, preferentially releases arachidonic acid from phosphoglycerides in a cytosolic Ca²⁺-concentration-dependent manner (Clark *et al.*, 1991; Shimizu *et al.*, 2006; Leslie *et al.*, 2010; Vasquez *et al.*, 2018; Astudillo *et al.*, 2019). Arachidonic acid generated by cPLA₂α serves as a precursor of pro-inflammatory eicosanoids, including certain prostaglandins and leukotrienes. Consequently, cPLA₂α-mediated bioactive lipid production plays a major regulatory role in physiological and pathogenic processes (Bonventre *et al.*, 1997; Uozumi *et al.*, 1997; Leslie, 2015). Gaining insights into cPLA₂α activation by divalent cation and lipid regulatory mediators are of great importance because arachidonic acid release by cPLA₂α at the membrane

surface is the rate-limiting step in eicosanoid production. Mechanistically, the membrane translocation of cPLA $_{2\alpha}$ is driven primarily by its N-terminal C2-domain rather than its catalytic domain (Clark *et al.*, 1991; Nalefski *et al.*, 1994 and 1998; Davletov *et al.*, 1998; Dessen *et al.*, 1999). Complexation of two Ca $^{2+}$ ions by the C2-domain neutralizes several Asp residues, facilitating protein docking and penetration into the membrane interface region (Davletov *et al.*, 1998; Perisic *et al.*, 1998; Xu *et al.*, 1998; Ball *et al.*, 1999; Bittova *et al.*, 1999; Dessen *et al.*, 1999; Malmberg *et al.*, 2003; Malmberg and Falke, 2005). Among lipids, ceramide-1-phosphate is an established regulator of the cPLA $_{2\alpha}$ action and C2-domain-driven membrane interaction (Pettus *et al.*, 2004; Stahelin *et al.*, 2007; Lamour *et al.*, 2009; Ward *et al.*, 2013). Previous studies of cPLA $_{2\alpha}$ C2-domain have relied on expression in *E. coli*, recovery of inclusion bodies, followed by the laborious task of refolding of recombinant protein prior to purification. Herein we describe a direct approach for obtaining purified C2-domain in soluble form. The approach utilizes established technology involving protein fusion with a cleavable 6xHis-SUMO tag to enable expression of soluble cPLA $_{2\alpha}$ C2-domain in *E. coli* followed by affinity purification (Malakhov *et al.*, 2004; Marblestone *et al.*, 2006; Yan *et al.* 2009; Peroutka *et al.*, 2011). An added advantage is that removal of the 6xHis-SUMO tag relies on Ulp1 protease recognition of SUMO tertiary structure, thus avoiding unspecific cleavage associated with other proteases that target a linear protein sequence. The protocol, which has been used to facilitate successful structural characterization of chicken C2-domain complexed with dihexanoyl phosphatidylcholine, also enables successful expression and purification of mouse, zebrafish, and human phospholipase A $_{2\alpha}$ C2-domains in soluble form (Hirano *et al.*, 2019).

Materials and Reagents

1. Amicon Ultra Centrifugal Filter (Millipore Sigma, catalog number: UFC901024)
2. *Escherichia coli* strain BL21 star (DE3) (Thermo Fisher Scientific, catalog number: C601003)
3. pET-SUMO vector
4. Chicken cPLA $_{2\alpha}$ C2-domain cDNA (synthesized by GENEWIZ)
5. cComplete[™] His-Tag Purification Resin (Millipore Sigma, catalog number: 5893682001)
6. HiTrap Q HP (Millipore Sigma, catalog number: GE29-0513-25)
7. Superdex 75 prep grade (Millipore Sigma, catalog number: S6657)
8. Isopropyl- β -D-thiogalactopyranoside (IPTG) (Thermo Fisher Scientific, catalog number: 34060)
9. Imidazole (Sigma-Aldrich, catalog number: 56748)
10. Glycerol (Thermo Fisher Scientific, catalog number: BP229-1)
11. Tris Base UltraPure (Research Products Intl., catalog number: T60040)
12. NaCl (Sigma-Aldrich, catalog number: 71376)
13. CaCl $_2$ (Sigma-Aldrich, catalog number: C7902)
14. Solution A (see Recipes)
15. Solution B (see Recipes)
16. Solution C (see Recipes)
17. Solution D (see Recipes)

18. Solution E (see Recipes)
19. Solution F (see Recipes)
20. Solution G (see Recipes)

Equipment

1. 250 ml and 2 L Erlenmeyer culture flasks
2. High speed centrifuge (Beckman Coulter)
3. Shaker incubator (Sanyo, orbital incubator)
4. Sonicator (Heat Systems-Ultrasonics Inc., model: W-225)
5. Benchtop centrifuge (Spectrafuge)
6. ÄKTA Purifier chromatography system (GE Healthcare)

Procedure

1. Transform *E. coli* strain BL21 star (DE3) cells with pET-SUMO vector containing the C2-domain open reading frame (residues 16-140) of human, chicken, mouse, or zebrafish cPLA $_{2\alpha}$.
 - a. Thaw one vial of competent cells on ice per transformation.
 - b. Add 5-10 ng of the plasmid in a volume of 1-5 μ l to the cells and mix by gently tapping.
 - c. Incubate the vial(s) on ice for 30 min.
 - d. Heat shock the cells by incubating the vial(s) for exactly 30 s in a 42 °C incubator and then remove the vial(s) from the 42 °C incubator and quickly place on ice.
 - e. Add 250 μ l of pre-warmed SOC medium to the vial(s) and then incubate the vial(s) at 37 °C for 1 h.
 - f. Plate the transformation reaction onto LB plates containing kanamycin (25 μ g/ml) for plasmid selection. Invert the plates and incubate at 37 °C overnight.

Notes:

i. Chicken cPLA $_{2\alpha}$ C2-domain sequences

Synthesized ORF sequence

TATAGCCATGTGTTTACCGTGACCGTGCGCAAAGCCACCAATGTGACCAAAGGCGCCATT
GGCGATATGCTGGATACCCCTGATCCTTATGTGGAGCTGTTTATTCCGAGCGCACCGGAT
TGCCGCAAACGCACCAAGCACTTCAACAACGACGTGAACCCGGTGTGGAACGAGACCTTC
GAGTTTATCCTGGACCCGAACCAGGATAACGTGCTGGAAGTGACCCTGATGGACGCCAAC
TATGTGATGGATGAGACCCTGGGCATGGCCACCTTCCGATTAGCAGCCTGAACTGGGC
GAGAAGAAAGAAGTTCAGCTGACCTTCAACAACGTGACCGAGATGACCCTGGAAGTGGAGC
CTGGAAGTTTGCAGC

Wild-type ORF sequence

TACTCACACGTATTCCTGTGACAGTAAGGAAAGCCACAAATGTCACAAAAGGAGCCATT

GGCGACATGCTTGACACTCCAGACCCCTATGTAGAGCTGTTTCATCCCTTCCGCTCCTGAC
TGCAGGAAGAGAACTAAACATTTCAATAATGATGTGAATCCTGTGTGGAATGAGACCTTC
GAATTCATTCTGGACCCCAACCAGGACTCTTGGTATGGCCAACAATGTTCTTGAGGTTAC
TTTGATGGATGCCAACTACGTTATGGATGAGCATTTCCTATATCCTCTTTGAAACTGGGA
GAGAAGAAGGAGGTCCAGTTAACTTTCAACAATGTTACAGAAATGACTTTGGAGCTGTCT
CTTGAAGTGTGCTCT

Chicken C2-domain Protein sequence coded by either ORF

YSHVFTVTVRKATNVTKGAIGDMLDTPDPYVELFIPSAPDCRKRTKHFNNVDNPNVWNETF
EFILDPNQDNVLEVTLMANYVMDETLMGMATFPISLKLGEKKEVQLTFNNVTEMTLELS
LEVCS

- ii. *In the synthesized chicken C2-domain ORF, codon usage is optimized for E. coli expression. BamHI and Sall restriction sites are used for open reading frame (ORF) ligation into a modified pET-28-SUMO vector (kanamycin-resistant). Prior to insertion, the human ORF requires mutation to remove a single BamHI internal restriction site 5'-¹⁹²GGATCC¹⁹⁷-3' without changing the protein sequence. The corresponding GGACCC sites in the chicken sequences are underlined.*

2. Collect 5-10 colonies and inoculate 20 ml of autoclaved LB medium (supplemented with 25 μ g/ml kanamycin) in a 250 ml Erlenmeyer culture flask. Incubate overnight at 37 °C while shaking at 200 rpm.
3. Use 10 ml of culture from Step 2 to inoculate 1 L of fresh LB medium containing 25 μ g/ml kanamycin and grow in a 2 L Erlenmeyer culture flask at 37 °C until OD₆₀₀ = 0.6-1.0.
4. Cool the resulting culture to 20 °C and add IPTG to yield a final concentration of 0.1 mM.
5. After 16-20 h of induction with IPTG at 20 °C and stirring at 220 rpm, collect the cells by centrifuging the culture at 5,000 $\times$ g for 10 min.
6. Suspend harvested cells in 30 ml of solution A and freeze at -30 °C until use.
7. Thaw frozen cells and disrupt by French press or sonication. When using probe sonication, apply 6 $\times$ 20 s pulses while keeping the sample (30 ml) in an ice bath to prevent heat damage.
8. Transfer the lysate to a centrifuge bottle and centrifuge at 25,000 $\times$ g for 30 min at 4 °C before loading the resultant supernatant onto a 3 ml column of cComplete His-Tag Purification Resin pre-equilibrated with Solution A.
9. Wash the resin with more than 10 resin-bed volumes using Solution B and then elute the protein with 10 ml of Solution C.
10. Add Ulp1 protease to remove the N-terminal 6xHis-SUMO tag.

Note: An advantage of the Ulp1 protease compared to other sequence-based proteases is the ability of Ulp1 to recognize SUMO tertiary structure and cleave at the C-terminal end of the conserved Gly-Gly sequence in SUMO. This ability eliminates unspecific cleavage associated with other proteases that target a linear protein sequence. SUMO cleavage by Ulp1 does leave two extra amino acids (Gly-Ser) at the N-terminal end of the C2-domain.

11. Dialyze the sample with 2 L of Solution D for more than 3 h at 4 °C.
12. Equilibrate an anion exchange chromatography column (HiTrap Q HP, 1 ml, 0.7 × 2.5 cm) with Solution E using a GE AKTA Prime Liquid Chromatography System.
13. Load the dialyzed solution from Step 11 onto the pre-equilibrated anion exchange column and elute using a 3 to 50% linear gradient of Solution F.
Note: The anion exchange chromatography (Steps 12 and 13) is optional. In Hirano et al. (2019), this chromatography approach was included for crystallography but not for fluorescence resonance energy transfer or surface plasmon resonance experiments since SDS-PAGE revealed similar very high purity in either case.
14. Combine the fractions containing cPLA $_2\alpha$ C2-domain and load onto cOmplete™ His-Tag Purification Resin (3 ml) pre-equilibrated with Solution B, wash the resin with 10 resin-bed volumes of Solution B and then elute the protein with 10 ml of Solution C.
15. Concentrate the flow-through using an Amicon Ultra-15 Centrifugal Filter (15 ml sample volume, 10 kDa cutoff) to less than 10 ml and load onto a HiLoad16/60 Superdex 75 pg gel filtration column (L x I.D.; 60 cm × 16 mm, GE Healthcare) pre-equilibrated with Solution G for final size exclusion purification of C2-domain monomer using Solution G (Figure 1). SDS-PAGE analysis shows the purity of the resulting monomeric protein (Figure 2).

Figure 1. Chromatogram of gel filtration purification (Step 15 in the Procedure). Y-axis represents absorbance at 280 nm whereas the X-axis represents elution time. The arrow corresponds to the elution peak for monomeric cPLA $_2\alpha$ C2-domain (16-140; 14.3 kDa).

Figure 2. SDS-PAGE analysis of purified chicken cPLA $_2\alpha$ C2-domain (16-140). The protein load for purified C2-domain equals 1-2 μ g.

16. Concentrate up to 20 mg/ml and store at -80 °C until use or add glycerol to a final concentration of 50% (v/v) and store at -20 °C. Typical yields of human C2-domain (1.3 mg) and chicken C2-domain (2.2 mg) are readily achievable from 2 L of starting culture medium.

Notes

Readers are referred to the methods section in Hirano *et al.* (2019) for additional details regarding application of the current approach to the purification of human cPLA $_2\alpha$ C2-domain.

Recipes

1. Solution A
20 mM Tris-HCl (pH 8.0)
500 mM NaCl
2.5 mM CaCl $_2$
2. Solution B
20 mM Tris-HCl (pH 8.0)
150 mM NaCl
2.5 mM CaCl $_2$
5 mM imidazole
3. Solution C
20 mM Tris-HCl (pH 8.0)
150 mM NaCl
2.5 mM CaCl $_2$
250 mM imidazole

4. Solution D
20 mM Tris-HCl (pH 8.0)
30 mM NaCl
2.5 mM CaCl₂
5. Solution E
20 mM Tris-HCl (pH 8.0)
2.5 mM CaCl₂
6. Solution F
20 mM Tris-HCl (pH 8.0)
1,000 mM NaCl
2.5 mM CaCl₂
7. Solution G
20 mM MES-NaOH (pH 6.0)
100 mM NaCl
2.5 mM CaCl₂

Acknowledgements

We are grateful for support received by the Program for Promoting the Enhancement of Research Universities from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan (YH); research grants from the National Institutes of Health via HL125353 (REB, DJP, and CEC), HD087198 (CEC) and RR031535 (CEC); the Veteran's Administration [VA Merit Review, I BX001792 (CEC)]; a Research Career Scientist Award 13F-RCS-002 (CEC); a Memorial Sloan-Kettering Cancer Center Core Grant P30 CA008748 (DJP); the Maloris Foundation (DJP); and the Hormel Foundation (REB). The contents of this manuscript do not represent the views of the Department of Veterans Affairs or the United States Government.

Parts of the current protocol, *i.e.*, use of SUMO tag, were adapted to enable expression of cPLA₂ α C2-domain in soluble form in *E. coli*. Original references pertaining to SUMO tag use include Malakhov *et al.*, 2004; Marblestone *et al.*, 2006; Yan *et al.* 2009 and Peroutka *et al.*, 2011.

Competing interests

The authors declare no competing interests.

Ethics

The current study involved no use or involvement of human or animal subjects.

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