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Orthogonal optimization of prokaryotic expression of a natural snake venom phospholipase A_2 inhibitor from *Sinonatrix annularis*

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ABSTRACT

Phospholipase A_2 (PLA_2) is a calcium-dependent enzyme that is involved in inflammatory processes such as the liberation of free arachidonic acid from the membrane pool for the biosynthesis of eicosanoids. Snake venom are known containing PLA_2s ($svPLA_2$s) which exhibit a wide variety of pharmacological effects including neurotoxicity, cardiotoxicity, myotoxicity and hemorrhage. Therefore, inhibition of $svPLA_2$ would be advantageous to successful envenomation treatment. A gamma type PLI (PLA_2 inhibitor) has been extracted from the serum of *Sinonatrix annularis*, a non-venomous snake indigenous to China. This showed strong inhibition of *Deinagkistrrodon acutus* PLA_2, however, the PLIγ level in the serum and snake resource are not sufficiently sustainable for further research. To overcome these limitations, we constructed a His6-PLIγ pET28 fusion expression vector and transformed *Escherichia coli* BL21. To improve the expression of PLIγ, an orthogonal experiment [$L_{16}(4)^5$] was performed to optimize induction parameters. The optimized condition was determined to be: induction by 0.4 mM isopropyl-β-d-thio-galactoside (IPTG) for 6 h to the recombinant BL21 after its OD_{600} was 0.8, with continuous shaking cultivation at 190 rpm and 35 °C. Under these conditions, the amount of expressed protein could reach 57 mg/L. The His6-PLIγ was purified by nickel affinity chromatography and renatured by On-column refolding. The resulting PLIγ showed a good inhibitory effect of enzymatic activities to venom PLA_2 isolated from *D. acutus*. Moreover, the PLIγ had a wide anti-hemorrhage activities to *D. acutus*, *Naja atra* and *Agkistrodon halys* venom.

1. Introduction

Snake venoms contain abundant phospholipase A_2s (PLA_2s) (E.C: 3.1.1.4), a kind of wide spread enzymes that are important in predation and digestion by vipers. Snake venom PLA_2s ($svPLA_2$) hydrolyze various phospholipids, releasing lysophospholipids and fatty acids. The hydrolysis increases cell permeability, which results in serious toxic reactions including cytotoxicity, bleeding, edema, hypotension and cascade inflammation (Gutierrez et al., 2006; Alirol et al., 2010; Gutierrez and Lomonte, 2013). Viperid and crotalid venom contain PLA_2s which can cause rapid necrosis of skeletal muscle fibers, thus being referred to as myotoxic PLA_2 (Harris and Cullen, 1990). To-date, all known pre-synaptic neurotoxins from snake venom are PLA_2 enzymes per se, or contain PLA_2 as an integral part (Bon, 1997; Faure et al., 2000). The high mortality and morbidity of snakebite envenomations are believed to be due to the various pathophysiological effects of $svPLA_2$s (Gutierrez and Lomonte, 2013). There are 383 $sPLA_2$ genes that have been discovered from 103 snake species, and 67 $svPLA_2$s or isoenzymes that have been studied and proved to be toxic (EXPASY protein database). Therefore, svPLA2s are major targets for the design and mining of anti-venom drugs.

Currently, multi-valence, anti-venom sera are the prevalent medicine for snakebite treatment. However, although they have been used for decades, their use can be problematic. Some of the difficulties include market shortage (Simpson and Blaylock, 2009), having side effects common to serum products (Leon et al., 2013; Deshpande et al., 2013), inconvenience of storage, and limited applications for snakebites. In China, there is only one antivenin manufacturer producing four antivenins to *Naja naja atra* (cobra), *Deinagkistrodon acutus* (hundred-pace snake), *Bungarus multicinctus* (coral) and *Agkistrodon halys* (viper). These existing antivenins are therefore applicable to only a limited number of

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venomous snakebites.

Based on these considerations, PLA_2 inhibitors are considered ideal candidates for anti-venoms and also for anti-inflammation caused by non-toxic PLA_2 in mammals (Marcussi et al., 2007). Scientists have made great efforts to extract chemical PLA_2 inhibitors from herbs, marine organisms and artificial chemical compounds (Carvalho et al., 2013). Interestingly, snakes are not sensitive to their own venom. The basis of self-protection is that snakes produce special PLA_2 inhibitors (PLI) in serum, which can block $svPLA_2$s enzymatic activity and/or neutralize their toxicity. PLIs expression was significantly up-regulated when *Gloydius brevicaudus* was injected intramuscularly with its own venom (Kinkawa et al., 2010), indicating the effective anti-venom activity of PLIs.

To-date, three types of snake PLIs have been found, i.e. PLI α, β and γ. PLIα and PLIβ show specific inhibition to group II acidic $svPLA_2$ whereas PLIγ has a broad spectrum of inhibition toward PLA_2s from venomous snakes, mammals, bees and others (Nishida et al., 2010; So et al., 2008). A novel PLIγ isolated from *Sinonatrix annularis* (Taxonomy ID:74368) serum (Genbank JN975878) strongly inhibited lecithin lysis activity of *D. acutus* venom PLA_2s in vitro (Chen et al., 2011). However, the PLIγ level in serum and snake resource is unsustainable for further research and drug development. To overcome these limitations, we developed the plasmid construction, induction optimization, purification and refolding of His6-PLIγ by the pET28/BL21 prokaryote express system.

2. Materials and methods

2.1. Enzymes, plasmids and chemicals

We used PLIγ gene (JN975878, cloned previously in our lab from the liver of *S. annularis*), restriction endonuclease enzymes *Nde*I and *Xho*I (NEB, Ipswich, MA, USA), mouse anti-6 × his monoclonal antibody and horseradish peroxidase-conjugated goat anti-mouse IgG, 2 × EasyTaq PCR SuperMix, 100 bp DNA marker (Trans Biotechnology Company, Beijing, China), Ni^{2+}-NTA resin, Polyacrylamide Gel Reagents, Clarity™ Western ECL Blotting Substrate (Bio-Lad, Hercules, CA, USA), anion exchange column source 30Q(GE Healthcare Life Sciences, Piscataway, NJ, USA), Coomassie blue G250, Gel extraction kit (Solarbio Bio-technology Company, Beijing, China) and snake venom PLA_2 (1 mg/ml) previously purified from *D. acutus*(Taxonomy ID:36307). Crude venom powder of *D. acutus*, *N. atra*, and *A. halys* were produced by Huangshan snake farm (Huangshan, Anhui province, China). Adult male Balb C mice are breed and cultured in Animal Center, Nanchang university.

2.2. Construction of expression vector of PLIγ

Based on the map of pET28c(+) vector and PLIγ gene sequence, a pair of specific primers was designed to amplify the PLIγ gene by PCR. The forward primer was 5′-GGCATATGATGCGCTCATGTGAAATTTGTCACAA-3′ with the *Nde*I restriction site underlined. The reverse primer is 5′-GGCTCGAGTTATTCAGAAGGTGTAGTTTTGG-3′ with the *Xho*I restriction site underlined. PCR amplification was conducted in 0.2 ml PCR tubes containing 25 μl of 2 × EasyTaq PCR SuperMix, 2 μl (10 μM) each of forward and reverse primers, 50 ng of cDNA and autoclaved Milli-Q water to make a volume up to 50 μl. Thirty-five cycles of amplification were run as follows: denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s. The PCR product was then digested by *Nde*I and *Xho*I, separated by agarose gel electrophoresis and recovered by using Gel extraction kit, and finally was inserted into pET28c (Fig. 1). According to the vector map, a 6 × His-tag was thus incorporated into the N-terminal of PLIã, leading to the fusion expression of His6-PLIã, which facilitated the subsequent affinity purification and Western blot determination. The formed vectors were transformed into competent *Escherichia coli* DH5α and verified by restriction enzyme digestion and sequencing. The recombinant constructs were then transformed into *E. coli* BL21 for PLIγ expression.

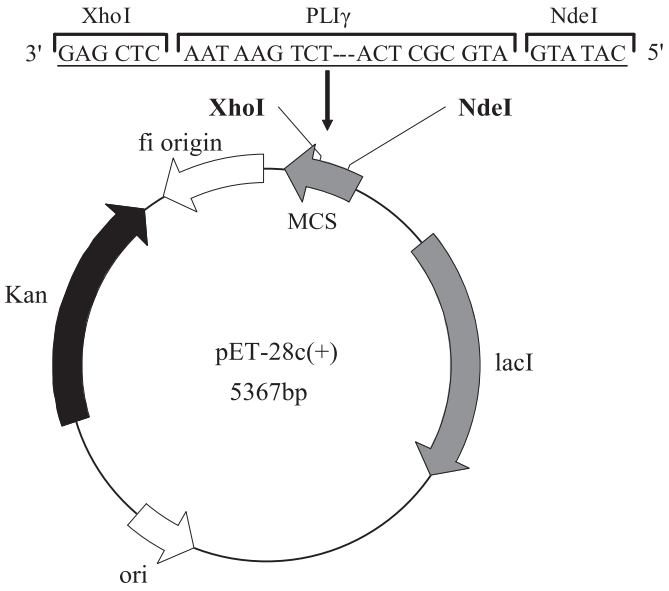

Fig. 1. Map of recombinant vector pET28c-PLIγ. *Nde*I and *Xho*I were the recombination sites.

2.3. Expression of His6-PLIγ

E. coli BL21 cells harboring the recombinant plasmid were inoculated in LB medium containing 50 μg/ml kanamycin at 37 °C overnight. This overnight culture was then transferred into 50 ml fresh LB medium at 1:50 dilution. The cells were grown at 30 °C, with constant shaking at 160 rpm. When the OD_{600} value of bacteria reached 0.4, the inducer IPTG was added into the culture to a final concentration of 0.6 mM. A parallel control was set without IPTG induction. After a 4 h fermentation at 37 °C, PLIγ expression was detected by sampling 1 ml of each group bacteria suspension and running SDS-PAGE and Western blot.

2.4. Optimization of culture conditions

To increase PLIγ production, an orthogonal test was performed to optimize the culture parameters of recombinant BL21. Five main factors can affect protein acumination in prokaryotic expression, i.e. temperature, shaker speed, OD_{600} (inducement time point), IPTG concentration and inducing time. An orthogonal table of $L_{16}(4)^5$ (Table 1) shows that each factor was tested at four different levels, which made 16 tests in total. When the 16 orthogonal trials were completed, 1 ml of bacteria from each group was sampled and determined by Western blotting (detected by His6 antibody). Basically, total bacteria protein was separated on 12% polyacrylamide gel and transferred onto polyvinylidencefluoride (PVDF) membrane at 0.4 A for 90 min. The working concentration of first antibody (mouse anti-His6) and second antibody (HRP-IgG) were 1:1000 and 1:10000, respectively. The recombinant PLIγ were visualized by ECL incubation and filmed in cassette. Protein quantification was done by Quantity One 4.6.2 software (http://www.bio-rad.com/en-us/product/quantity-one-1-d-analysis-software,