Expression, Purification, Refolding and Characterization of A *Neverland* Protein from *Caenorhabditis Elegans*

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Research

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Abstract

Background: Steroid hormones serving as vital compounds are required for a variety of organisms' development and metabolism. The *neverland* (NVD) family genes encode conserved Rieske-type oxygenases, which are accountable for the dehydrogenation during the synthesis and regulation of steroid hormones. However, the His-tagged NVD protein from *Caenorhabditis elegans* expresses as inclusion bodies in *E. coli*/BL21(DE3). This neck bottle can be solved through refolding by urea or introduction of maltose-binding protein (MBP) tag at N-terminus.

Results: Further research on purification after introduction of maltose-binding protein (MBP) tag at N-terminus, CD measurement and fluorescence-based thermal shift assay indicated that MBP was favorable for the NVD proteins' solubility and stability, which may be beneficial for large-scale manufacture of NVD protein for further research. The structural model contained Rieske [2Fe-2S] domain and non-heme iron-binding motif, which were similar with 3-ketosteroid 9 α -hydroxylase.

Conclusions: The successful introduction of maltose-binding protein (MBP) tag at N-terminus could increase the soluble expression of CeNVD, is advantageous for further purification and improvement of thermostability. Our study provides a persuasive case for soluble expression of Rieske-domain oxygenase in *E. coli*.

Background

Biotransformation Sterols derivatives mediate a wide range of growth, development and evolution in most living species [1]. In insects, the steroid hormone ecdysone played an essential role in developmental transitions and egg production [2]. The flies could not reach the adult stage when disable for synthesis of lathosterol by shutting down the NVD gene using RNA interference (RNAi) *in vivo*. This matter can be solved by supplementing standard food or lathosterol on time [3]. The sterol metabolites also had many important properties mostly related to the biosynthesis and regulation of amino acids and vitamins [4], which involve in cholesterol homeostasis and synthesis of vitamin D₃.

The metabolites of vitamin D₃ (cholecalciferol) have raised great concern due to its biological effects and physiological properties, such as calcium metabolism and phosphate homeostasis, regulation of immune responses, promotion of insulin secretion and stimulation of cell proliferation and differentiation [5]. The vitamin D₃ was synthesized in humans and most of vertebrate animals on the skin, in which the 7-dehydroxycholesterol (7-DHC) was converted into pre-vitamin D₃ via ultraviolet (UV) irradiation at wavelengths of 290–320 nm, and meanwhile followed by a thermal isomerization to form vitamin D₃ spontaneously [6]. The high incidence of renal bone disease, osteomalacia and osteoporosis were reported to be associated with malabsorption of calcium, which caused by deficiency of vitamin D₃ [7]. Compared with chemosynthesis of 7-DHC using cholesterol [8], the bioconversion of cholesterol into 7-DHC has attracted more attention with regioselectivity and no pollution for environment [3, 9–11]. The reaction can be catalyzed by the evolutionarily conserved Rieske-domain oxygenase *neverland* (NVD),

which contains a Rieske [2Fe-2S] cluster binding domain to function as electron acceptor and electron transfer and a highly conserved non-heme iron-binding center as catalytic domain [9]. Several Rieske-domain oxygenase genes from reptiles, insects, nematodes and deuterostome species had been reported including *Anolis carolinensis* (protein ID XP_003230725.2), *Anopheles gambiae* (protein ID EAA04927.5) [12], *Bombyx mori* (protein ID BAE94192.1) [13], *Caenorhabditis elegans* (protein ID CAA98235.2) [14], *Ciona intestinalis* (protein ID BAK39961.1) [11], *Drosophila melanogaster* (protein ID ABW08586.1) [15], *Danaus plexippus* (protein ID OWR46621.1) [16], *Danio rerio* (protein ID BAK39960.1) [9], *Gallus gallus* (protein ID XP_425346.2) [9], *Hemicentrotus pulcherrimus* (protein ID BAK39963.1) [11], *Rhodococcus rhodochrous* (*kshA*, protein ID ADY18310.1) [17], *Spodoptera littoralis* (protein ID ADK56283.1) [18], *Pseudomonas fluorescens* (*prnD*, protein ID AAB97507.1) [19], *Podarcis muralis* (protein ID XP_028576239.1), *Pseudonaja textilis* (protein ID XP_026568371.1), and *Xenopus laevis* (protein ID BAK39959.1) [11]. The family proteins are essential regulator of cholesterol metabolism and steroidogenesis.

In addition, NVD from *Caenorhabditis elegans* (CeNVD) were identified in metabolic pathway of cholesterol, and genetic evidence has demonstrated that the NVD gene plays a vital role in larval development and adult aging in ecdysteroid biosynthesis. [11, 14] However, there have been few reports about the effective heterogeneous expression and production system of NVD family proteins in vitro. Here, we verified that the NVD protein was expressed as inclusion bodies with His-tag [20], and a small amount of soluble protein was obtained, even though it was further refolded by urea. Subsequently, we introduced maltose-binding protein (MBP) to enhance the soluble expression and purification of CeNVD in *E. coli* BL21(DE3), and the thermostability of CeNVD was also improved.

Materials And Methods

Materials

The *neverland* gene from *Caenorhabditis elegans* (CeNVD) was chemically synthesized in pET-28a(+) (Novagen, Madison, WI, USA) vector by GENEWZ (Suzhou, China) after codon-optimized. The DNA fragment of 1110 bp was PCR amplified using gene specific primers contained the *EcoR* I and *Hind* III restriction sites at the 5'- and 3'-terminal, and was cloned into pMal-c2X (New England Biolabs, Beverly, MA, USA) plasmid vector which contains a N-terminal MBP-tag and sequenced. The *E. coli* BL21 (DE3) (Novagen, Darmstadt, Germany) strain was employed as heterologous expression host.

Expression and purification

The recombinant plasmid was transformed into *E. coli* BL21(DE3) strain and grown in Luria-Bertani (LB) medium supplemented with kanamycin (50 µg/ml) or ampicillin (100 µg/ml) with a shaking of 220 rpm at 37 °C. When the optical density at 600 nm (OD₆₀₀) reached 0.6-0.8, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture and the recombinant cells were cultivation with a shaking of 160 rpm at 16 °C for 16-18 h to induce the protein expression. After cultivation, the

recombinant cells were harvested by centrifugation at 5,000 $\times g$ for 15 min at 4 °C, washed twice with PBS (pH 8.0) [21].

In order to purify the CeNVD_pET-28a(+), the washed cells were resuspended in 30 mL lysis buffer A (20 mM Tris-HCl, 20 mM imidazole, 500 mM NaCl, and 1 mM dithiothreitol, pH 8.0) containing 0.5 mg/mL lysozyme and 1 mM phenylmethane sulfonyl fluoride (PMSF) and disrupted using a sonicator (Sonic Dismembrator Model 100, Pittsburgh, PA) on ice bath for 20 min, the unbroken cells and cell debris were removed by centrifugation at 20,000 $\times g$ for 30 min at 4 °C, the supernatant was applied to a nickel-nitrilotriacetic acid (Ni-NTA) agarose affinity chromatography matrix (Qiagen, Hilden, Germany), pre-equilibrated with lysis buffer A, after washing the open column with 10 mL lysis buffer A extensively, the bound protein was eluted with 10 mL elution buffer A (20 mM Tris-HCl, 300 mM imidazole, 300 mM NaCl, and 1 mM dithiothreitol, pH 8.0) [22].

For purification of CeNVD_pMal-c2X, the amylose resin was applied to fixed MBP_CeNVD fusion protein. The unbound protein was washed with lysis buffer B (20 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol, pH 8.0), and the target protein was eluted with 10 mL elution buffer B (20 mM Tris-HCl, 20 mM maltose, 500 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol, pH 8.0) [20]. Then the protein was further purified by anion exchange chromatography employed a Resource Q column (column volume: 6 mL, flow rate: 4 mL/min, GE Healthcare, Stockholm, Sweden) on ÄKTA system (GE Healthcare, Sweden) [23]. The purified enzyme was eluted with a linear gradient between 0 to 1 M NaCl at a flow rate of 3 mL/min. Subsequently, the MBP tag was digested using a Factor Xa Protease (New England Biolabs, Beverly, MA, USA) at 4 °C for 12 h, and then loaded on amylose resin to remove the MBP tag and undigested protein. The flow through buffer contained target protein was collected and concentrated for further experiments.

Western blot analysis of CeNVD_pET-28a(+)

After centrifugation of the disrupted CeNVD_pET-28a(+), the cleared supernatant and precipitant were loaded on SDS-PAGE gels, then all protein molecules was transferred to a PVDF membrane and blocked in PBST buffer (PBS pH 8.0, 0.02% Tween-20) containing 1% bovine serum albumin (BSA) for 2 h, followed incubation in anti-His-tag mouse monoclonal antibody (Abcam, Cambridge, UK) which was diluted in blocking buffer (PBST pH 8.0, 1% BSA) at the indicated concentrations of 1:5000 for 12 h at 4 °C. After washing with PBST for 4 times, the membrane was protected from light and incubated with HRP-conjugated secondary antibody (HRP-conjugated goat anti-mouse IgG, Tiangen Biochemistry, Beijing, China) at a dilution of 1:1000 and room temperature for 2 h. After washing, the target protein was trapped by HRP-DAB chromogenic substrate kit (Tiangen Biochemistry, Beijing, China) and the immunoreactive band was digitally scanned using an Odyssey Infrared Imager (LI-COR Bio-science, Lincoln, NE, USA) [24].

The refolding of denatured protein

The CeNVD_pET-28a(+) cells were collected and resuspended in lysis buffer C (20 mM Tris-HCl, 20 mM imidazole, 500 mM NaCl, 8 M urea and 1 mM DTT, pH 8.0), and then disrupted and centrifuged as mentioned above. The cleared supernatant containing the denatured enzyme was refolded by sequential dialysis with gradient descent of urea concentrations (7, 6, 5, 4, 3, 2, 1, 0.5, 0 M) and then trapped on pre-equilibrated Ni-NTA superflow resin (Qiagen, Hilden, Germany), washed with lysis buffer D (20 mM Tris-HCl, 20 mM imidazole, 500 mM NaCl, 1 mM dithiothreitol, 0.5 mM GSSG, 3 mM GSH, and 500 mM arginine, pH 8.0). The refolded protein was eluted with elution buffer D (20 mM Tris-HCl, 300 mM imidazole, 300 mM NaCl, 0.5 mM GSSG, 3 mM GSH, 500 mM arginine, and 1 mM dithiothreitol, pH 8.0) [25]. The protein concentration of each purification step was measured via the BCA protein assay kit (Solarbio, Beijing, China) [26].

Molecular mass determination

The molecular weight of native CeNVD was measured by gel filtration chromatography using a Superdex200 Increase 10/300 GL column on ÄKTA system (GE Healthcare, Sweden) [27]. The target enzyme was eluted with a buffer (20 mM Tris-HCl, 150 mM NaCl and 1 mM DTT, pH 8.0) at a flow rate of 1 mL/min with aldolase (158 kDa), conalbumin (75 kDa) as calibration proteins (GE Healthcare).

Circular dichroism (CD) measurements

The CD spectra was determined using a MOS-450 CD spectropolarimeter (Biologic, Claix, Charente, France). The protein sample was loaded into a 1 cm path-length quartz cuvette in which 0.1 mg/mL protein was dissolved in PBS (pH 8.0), the CD data were recorded in the far-UV band of 190-250 nm at room temperature for average of four times scan with a rate of 1 nm/s, a bandwidth of 0.1 nm and a step resolution of 0.1 nm. Analysis of the protein secondary structure was performed with the program BeStSel (<http://www.dichroweb.cryst.bbk.ac>) [28].

Fluorescence-based thermal shift assay

The thermal stability of CeNVD was characterized via fluorescence-based thermal shift assay using a 48-well assay plate real-time PCR instrument (Bio-Rad, Hercules, CA, USA). Reaction samples were conducted in three replicates that contained 0.4 mg/mL protein and 100×SYPRO Orange dye in PBS buffer (pH 8.0), The temperature was increased with a linear gradient of 20 to 90 °C at 0.5 °C/30 s and the minimal value was regard as the melting temperature (T_m) [29].

Structure modeling of CeNVD

Steered The three-dimensional (3D) homology model of CeNVD was generated by the SWISS-MODEL (swissmodel.expasy.org/) [30] using a template of 3-ketosteroid 9 α -hydroxylases from *R. rhodochrous* (PDB ID: 4QDF, 2.43 Å) [31], which shared 30% sequence identity with CeNVD. Then the generated models were visualized and analyzed using the PyMol software (<http://www.pymol.org>).

Results And Discussion

Sequence alignment and phylogenetic analysis

The phylogenetic tree of Rieske oxygenase from various microorganisms revealed that the evolutionary relationship of CeNVD was similar to that of *C. intestinalis* (Fig. 1A) with 41.8% amino acids sequence identity, it showed the lower sequence identity of 30.2% with *B. mori*. BLAST and sequence analysis indicated that the NVD from *C. elegans* shared higher sequence identity with *X. laevis* (45.7%), *D. rerio* (44.9%), *G. gallus* (44.0%), *C. intestinalis* (41.8%) and *A. gambiae* (40.3%). Amino acid sequences alignment and analysis with homologous proteins displayed that the family proteins contained two evolutionally conserved domains, Rieske [2Fe-2S] domain (C-X-H-X16-17-C-X2-H) and non-heme iron-binding motif [Fe(II); E/D-X3-D-X2-H-X4-H]. The conserved residues in CeNVD contained C122, H124, C143, H146 in the Rieske domain and E230, D234, H237, H242 in the Fe(II) domain (Fig. 1B).

Heterologous expression and purification of CeNVD recombinant enzyme

The CeNVD_pET28a(+) was expressed in *E. coli* BL21 (DE3) and purified by His-trap affinity chromatography. SDS-PAGE and western blot analysis demonstrated that the target protein appeared as a single band with a molecular mass of approximately 42 kDa, consistent with the calculated molecular weight of 42,800 Da. However, the protein was overwhelmingly expressed as inclusion bodies (Fig. 2A and 2B). Subsequently, the CeNVD gene was cloned and inserted into pMal-c2X, the reconstructed enzyme was overexpressed and purified by generic multiple-step purification using amylose fast flow resin and anion exchange chromatography (Fig. 3A and 3B). The MBP-tag was then digested by a Factor Xa protease and removed by amylose resin (Fig. 3C). The yields and purities of CeNVD for different purification stages are summarized in Table 1. Finally, 4.1 mg of CeNVD with 91.1% high purity was obtained in 200 mL of cell culture. Therefore, the MBP was conducive to the soluble expression and purification of CeNVD in *E. coli*, and the purification multiple-step purification method was necessary to obtain highly purified recombinant CeNVD.

Characterization of refolded His_CeNVD

His_CeNVD was expressed as inclusion bodies. Therefore, we added arginine and urea to refold the protein with extra redox system to improve the refolding yield, such as reduced and oxidized glutathione (GSH and GSSG) [32]. Here, we used gradient descent of urea with 500 mM arginine and a redox pair (GSH and GSSG) to facilitate the protein solubilizing and refolding. After dialysis, the refolded His_CeNVD was further purified by Ni-NTA affinity chromatography (Fig. 2C), and the fraction was further analyzed through gel filtration chromatography and a single peak was detected at 280 nm. Consistent with gel filtration chromatography analysis, SDS-PAGE indicated that the refolded His_CeNVD of higher purity (88.9%) (Table 1) was obtained and a trimer state in solution (Fig. 2D).

Characterization of all CeNVD

The secondary structure of MBP_CeNVD, ΔMBP_CeNVD and refolded His_CeNVD were measured by CD spectroscopy (Fig. 4A). The MBP_CeNVD showed a negative absorption peak centered around 202 nm, the percentage of α-helix, β-strand, turn, and unordered regions were 9.5 %, 24.7 %, 8.5% and 57.3 %, respectively. The CD spectrum of ΔMBP_CeNVD and refolded protein demonstrated a visibly increase of α-helix (22.5%, 16.4%), and a slightly decrease of turn structures (4.6 %, 6.7%), and unstructured regions (45.8 % and 51.6 %) (Table 2).

Fluorescence-based thermal shift assay was used to determine the thermostability of three type of CeNVD, as showed in (Fig. 4B), the T_m value of MBP_CeNVD was higher than His_CeNVD and ΔMBP-NVD (52.5 °C, 50 °C and 48 °C), which suggested that MBP was profitable for the thermostability of CeNVD.

Structural analysis of CeNVD

The structure of CeNVD contains 6 α-helices (α1-α6) and 16 anti-parallel β-strands (β1-β16) (Fig. 5A and 5B), which are conserved in NVD family enzymes. It contains a N-terminal Rieske [2Fe-2S] cluster (C122, H124, C143, H146) and followed by the catalytic domain harboring non-heme Fe(II) center (E230, D234, H237, H242). The Rieske cluster was located at strands β4, β5 and β6, β7. As for the Fe(II) center, E230 and D234 are located at α2, H237 is located at η3, H242 is located on loop between η3 and β11 (Fig. 5D). Structural alignment revealed that the structure and residues in the major domains are remarkably similar to that of 3-ketosteroid 9 α-hydroxylases from *R. rhodochrous* (C67, H69, C86, H89) and (D174, D178, H181, H186) (Fig. 5C) [31].

Table 1
Summary of the yields and enrichment factors of CeNVD for different purification process.

Purification step	Total protein (mg)	Target protein (mg)	Purity (%)	Yield (%)
Supernatant of MBP_CeNVD	72.3	21.4	29.6	100
Eluate from Resource Q	12.6	9.7	77.0	45.3
Flow through from MBP-trap after digestting the MBP-tag	4.5	4.1	91.1	19.2
Eluate from Ni-NTA resin after refolding	8.7	6.4	73.6	29.9
Eluate from Superdex200	3.6	3.2	88.9	15.0
The results are based on cell material from a 200 mL <i>E. coli</i> culture.				

Table 2
Secondary structure assignments (%) of CeNVD determined by CD spectroscopy in the wavelength region from 190 to 250 nm

Protein	α -Helix	β -Strand		Turn	Other
		Antiparalle	Parallel		
Refolded His_CeNVD	16.4	11.9	13.4	6.7	51.6
MBP_CeNVD	9.5	8.9	15.8	8.5	57.3
Δ MBP_CeNVD	22.5	17	10.1	4.6	45.8

Conclusions

Our study provides a persuasive case for soluble expression of Rieske-domain oxygenase in *E. coli*. We successfully expressed and purify the CeNVD protein in *E. coli* with the soluble formation of refolded His-NVD and MBP-NVD, showing that MBP tag could increase the soluble expression of CeNVD, which is advantageous for further purification and improvement of thermostability.

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Declarations

Author contributions

FLu and H-MQ designed the research; SM, ZS, MW and XW performed the experiments and analyzed data; H-MQ, SM and FLu wrote the paper. All authors read and approved the final manuscript.

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Competing financial interests

No competing financial interests.

Availability of data and materials

The data collected upon which this article is based upon are all included in this manuscript and its additional information files.

Ethics approval and consent to participate:

No animals or human subjects were used in the above research.

Consent for publication

Our manuscript does not contain any individual data in any form.

Competing interests

No competing interests.

Figures

Figure 1

Sequence alignment and phylogenetic tree of CeNVD with family enzymes. The phylogenetic analysis of Rieske oxygenases from different species (A); Multiple alignment of CeNVD with other Rieske oxygenases (B), green triangle (▲) and orange asterisk (*) were responsible for the Rieske [2Fe-2S] domain (C-X-H-X16-17-C-X2-H) and non-heme iron-binding motif [Fe(II); E/D-X3-D-X2-H-X4-H] respectively, the alignment was prepared using the program ESPrift 3.0 service (<http://esprift.ibcp.fr/ESPrift/ESPrift/>).

Figure 2

Purification of CeNVD₁₋₁₁₆-pET28a(+) by Ni-NTA affinity chromatography. SDS-PAGE analysis of CeNVD₁₋₁₁₆-pET28a(+) (A); Western blot analysis of CeNVD₁₋₁₁₆-pET28a(+) (B); SDS-PAGE analysis of denatured and refolded protein (C); Size-exclusion chromatography of refolding protein, aldolase (158 kDa), conalbumin (75 kDa) as reference proteins (D); Lanes 1: supernatant; 2: sediment; 3: flow-through; 4: wash buffer; 5: resin before eluting; 6: elution buffer; 7: resin after eluting.

Figure 3

Purification of CeNVD_pMal-c2X by MBP-trap affinity chromatography (A); Purification of CeNVD_pMal-c2X by anion-exchange (B); Purification of CeNVD by MBP-trap without MBP tag (C).

Figure 4

The CD spectra (A) and melting curves (B) of CeNVD. The experiments were conducted in three replicates, and the data represent the means $\pm$ standard deviations.

Figure 5

The structure model of CeNVD (A). Monomer structure of CeNVD with the α -helices (cyan) and β -strands (salmon) are labeled (B); The superimposed subunits of CeNVD (blue) and 3-ketosteroid 9 α -hydroxylases from *R. rhodochrous* (yellow) with major domains. Residues are shown as green and magenta sticks; The stereo view of Rieske [2Fe-2S] domain and non-heme Fe(II) binding domain (D).