# Recombinant Protein Production of Von Willebrand Factor Type D Domain of *Fenneropenaeus Merguiensis* Vitellogenin

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## Abstract

The low density lipoprotein receptor (LDLR) family is a group of proteins that have several biological functions. Among their functions, the proteins in LDLR family are involved in lipoproteins trafficking. In the previous study, we found that the C-type lectin containing LDLR domain named FmLdlr from banana shrimp (*Fenneropenaeus merguiensis*) could interact with vitellogenin (Vg), the crucial protein for oogenesis in the shrimp. However, Vg is a multidomain apolipoprotein. There are three domains found in *F. merguiensis* Vg sorted by N-terminus to C-terminus containing lipoprotein N-terminal domain (LPD_N), domain of unknown function (DUF1943), and von Willebrand factor (vWF) type D domain (VWD). According to these information, we want to identify whether domain of Vg has the ability to interact with FmLdlr. Here we cloned and produced recombinant protein of VWD which is one of the domains in Vg of *F. merguiensis*. Firstly, we extracted the total RNA from hemocytes of the shrimp. Then, the total RNA was converted into first strand cDNA by *in vitro* reverse transcription. The first strand cDNA was used as a template for polymerase chain reaction (PCR) using VWD specific primers. The length of VWD PCR product was 459 bp. Then, the complete cDNA of VWD was cloned into cloning vector and transferred to pFN2A (GST) Flexi® vector. *Escherichia coli* strain BL21 Star™ (DE3) was used as an expression host. The result showed that the recombinant protein was successful expressed in bacterial system. The recombinant protein of VWD was expressed with a fusion protein of Glutathione S-transferase (GST). The molecular weight of the protein was estimated by using the relative migration distance (Rf) of standard proteins in SDS-PAGE was 39.81 kDa.

***Keywords*****:** *Vitellogenin, von Willebrand factor type D domain, fenneropenaeus merguiensis, recombinant protein*

## 1. Introduction

At present, shrimp is the most valuable species in the seafood industry worldwide. The shrimp culturing industry has been driving economic growth in Thailand and many countries in Southeast Asia for several years. The two species which are popular in Thailand shrimp culturing industry are *Penaeus monodon* and *Litopenaeus vannamei* (Wyban, 2007). *Fenneropenaeus merguiensis* (Banana shrimp) is an alternative cultured species. Since this specie is popular to cook for premium seafood dishes, it is an interesting specie to culture for commercial reasons. The major problem in the shrimp culturing industry that is causing economic loss has been the infectious diseases. To solve this problem, many studies concerning the shrimp immune system and the immunological molecules in the system were published. In *F. merguiensis*, we found the effective molecule named FmLdlr which belong to C-type lectins (CTLs) family. Our recently study demonstrated that FmLdlr was a CTL containing carbohydrate recognition domain (CRD) and low density lipoprotein receptor domain (LDLR). The CRD of FmLdlr was a major domain that could interact with bacterial and viral pathogens of shrimp and stimulate many immune mechanisms such as phagocytosis and encapsulation (Kwankaew et al., 2018) whereas LDLR had almost no activity to induce a shrimp immune response (Kwankaew et al., 2018). Interestingly, FmLdlr and its individual domains could bind to Vg. The domain within FmLdlr which could bind to Vg with the highest affinity was LDLR (Kwankaew et al., 2018). Vg is an egg yolk precursor protein found in the females of nearly all oviparous species (Meusy & Payen, 1988). It is an essential nutrient source for oocyte development (Valle, 1993). In crustacean including shrimp, Vg was produced abundantly by hepatopancreas of female and released into hemolymph to accumulate in ovary. In the journey from the

hepatopancreas to ovary, Vg required Vg receptor (VgR). The VgR is a member of the LDLR superfamily. However, the complete amino acid sequence analysis in all cases of Vg reveal that the protein contains three conserved domains (Sun et al., 2013) including the Vg_N domain at the N-terminal region, the domain of unknown function (DUF) 1943, and the von Willebrand factor type D domain (VWD) which is located at the C-terminus. No one knows the position or which domain in Vg that Fmldlr could bind to. In this work, we try to clone and produce the recombinant protein of VWD from hemocyte of *F.merguiensis* to use as the sample for the further study of interaction between Vg domains and FmLdlr.

## 2. Objectives

1. To clone VWD cDNA from hemocyte of *F.merguiensis*
2. To produce the recombinant protein of VWD

## 3. Materials and Methods

3.1 Shrimp

The mature female *F. merguiensis* were obtained from Nakhon Si Thammarat province, Thailand. These shrimp were cultured in aerated sea water and fed with pellet food four times a day until their body weight was around 15-20 g before preparing to the experiment.

3.2 Total RNA extraction

Hemocytes of *F. merguiensis* were obtained from shrimp by a sterile technique and then TriPure isolation reagent (Roche Diagnostics, Germany) was added in a ratio of 100 mg of tissue per 1 ml of TriPure isolation reagent. After homogenization, each sample was incubated 5 min at room temperature (waiting for nucleoprotein complexes separation) and centrifuged at 12,000xg for 10 min at 4°C to discard the tissue remnant or cell debris. Then the supernatant was transferred to a new tube. Afterwards, the chloroform was added and the suspension was mixed vigorously by vortex for 20 seconds with 15 min incubation at room temperature. After centrifugation at 12,000xg for 20 min at 4°C, the solution was separated and the total RNA in aqueous phase was transferred to a new tube and precipitated by incubation with an equal volume of isopropanol at -80°C for 2 h. The precipitated RNA was collected by centrifugation at 12,000xg for 20 min at 4°C and washed with 75% ethanol. The pellet was air dried. To dissolve the RNA pellet, RNase-free water was used and the RNA solution was incubated for 15 min at 55°C. Finally, RNA quality and concentration were finally measured using MeastroNano spectrophotometer (Meastrogen, USA) for the absorbance (optical density, O.D.) at 260 nm with the conversion factor 1 O.D.260 = 40 µg/ml RNA.

3.3 cDNA synthesis

To eliminate the contaminated DNA in the total RNA (1 µg) template, DNase I was added. After incubation at 37°C for 30 min, DNase I was inactivated using 1 µl of 25 mM EDTA (ethylenediaminetetraacetic acid) and heated for 10 min at 65°C. Total DNase-treated RNA was used as template for cDNA synthesis by SuperScript III RT (reverse transcriptase). The last process of cDNA synthesis was to remove RNA template from cDNA:RNA hybrid molecule by RNase H.

3.4 Cloning of a VWD cDNA

To clone VWD cDNA, the specific primers of VWD (Table 1) were designed based on the cDNA complete sequence of *F. merguiensis* Vg (GenBank: FJ644568.1). The reverse transcription polymerase chain reaction (RT-PCR) was used to amplify VWD gene. The PCR products were analyzed in 1% agarose gel electrophoresis and the expected band was purified by GenepHlow™ Gel/PCR Kit. The purified PCR product was cloned into a cloning vector (pGEM®-T Easy vector, Promega, USA), and transformed to *E. coli* strain DH5-alpha and sequenced.

3.5 VWD recombinant protein production

The RT-PCR was operated using specific primers of VWD containing SgfI and PmeI sites (Table 1). The pGEM-T Easy vector containing VWD gene was used as a template. The purified PCR product of VWD containing SgfI and PmeI sites was cloned into pGEM®-T easy vector, then the plasmid was digested with Flexi® Enzyme Blend (SgfI & PmeI) (Promega) and ligated into the pFN2A GST Flexi Vector (Promega) using DNA T4 ligase (Roche Diagnostics). The recombinant plasmid was rechecked for nucleotide sequence and transformed into the expression host, *E. coli* strain BL21 Star™ (DE3). The bacteria containing expression vector of VWD were incubated at 37 °C overnight (Figure 1). The cells were diluted at a ratio 1/100 and incubated at 37 °C for 4 hours to get O.D.600 of 0.4. After *E. coli* strain BL21 Star™ (DE3) containing the expression vector of VWD was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubation at 37 °C for 4 h, the bacterial cells were harvested and analyzed in 12% SDS-PAGE. Afterwards, the bacterial cells were sonicated for 2 min 5 times in lysis buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole). The insoluble protein was obtained after centrifugation at 10,000xg at 4°C for 30 min. The remaining sediment was solubilized in solubilized buffer (100 mM $NaH_2PO_4$, 10 mM Tris-base, 8 M urea) overnight. Finally, the expressed proteins were refolded by dialysis in a refolding buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 5 mM glutathione (GSH) reduced form, 0.5 mM Glutathione disulfide (GSSG) oxidized form) overnight.

**4. Results and Discussion**

First of all, cDNA from various tissues of *F. merguiensis* was used as a template for VWD domain amplification. Then, the complete cDNA of VWD was successfully cloned from hemocyte of *F. merguiensis*. The VWD domain fragment was sequenced. The nucleotide sequence of VWD domain isolated from hemocytes was identical to VWD domain of FmVg cloned from hepatopancreas (Puengyam et al., 2013) (Figure 3). The length of cDNA sequence is 459 bp (Figure 2) which translated to a peptide 153 amino acids. The calculated molecular weight of VWD protein using Vector NTI Advance® software (Thermo Fisher Scientific, USA) was 15,961 Da. The calculated pI was 8.03. The GST-tagged fusion VWD proteins was 386 amino acids in length. The molecular weight of GST-tagged fusion VWD indicated by relative migration distance value (Rf) was 39,810 Da (Figure 4). Its calculated pI using ExPASy tool was 6.57. Although, most studies in shrimp reported that the transcriptional expression of Vg was found in hepatopancreas and ovary. In this study we could clone the VWD domain from hemocytes. Human VWD was synthesized in endothelial cells and megakaryocytes (Kanaji et al., 2012) which are kinds of cells in the blood system. In this study, we inferred that VWD domain was present in the hemocyte protein and also in the mature Vg.

**Table 1** Sequences of primers used for VWD amplification

| Usage | Primer name | Sequence |
|---|---|---|
| VWD cDNA | VWD-F | 5'ATTACGGACGTGCTACCC 3' |
| | VWD-R | 5'ACCACTGGGCATGAGAC 3' |
| VWD SgfI restriction site | SgfI VWD-F | 5'GCGATCGCGATTACGGACGTGCTA3' |
| VWD PmeI restriction site | PmeI VWD-R | 5'GTTTAAACTCAACCACTGGGCATGA3' |

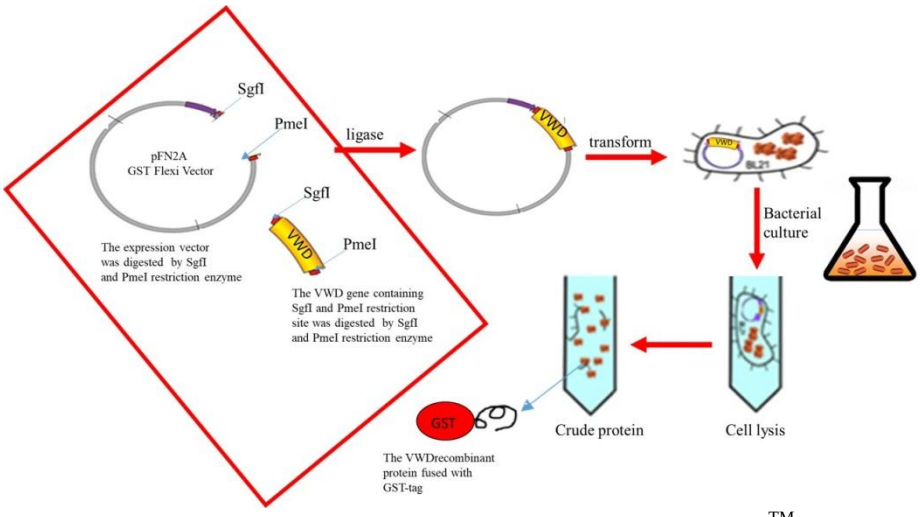


**Figure 1** The VWD recombinant protein production by *E. coli* BL21 Star™ (DE3)

In the production of VWD recombinant protein, the expression vector (pFN2A GST® Flexi vector) and the VWD gene containing SgfI and PmeI restriction sites was digested by SgfI and PmeI restriction enzymes. Then, they were ligated together and the expression vector containing VWD was transformed into the bacterial expression host. After the bacterial hosts were cultured, the bacterial cells were lysed and the over-expressed recombinant protein was found in the inclusion body.

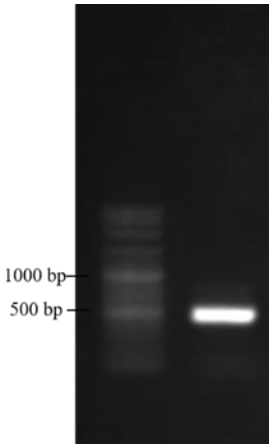


**Figure 2** The result VWD gene amplification from hemocytes in *F. merguiensis* using RT-PCR.
Left lane: VC 100bp Plus DNA Ladder (Vivantis Technologies, Malaysia).
Right lane: VWD cDNA (459 bp) in RT-PCR product using hemocytes of *F. merguiensis* as a template

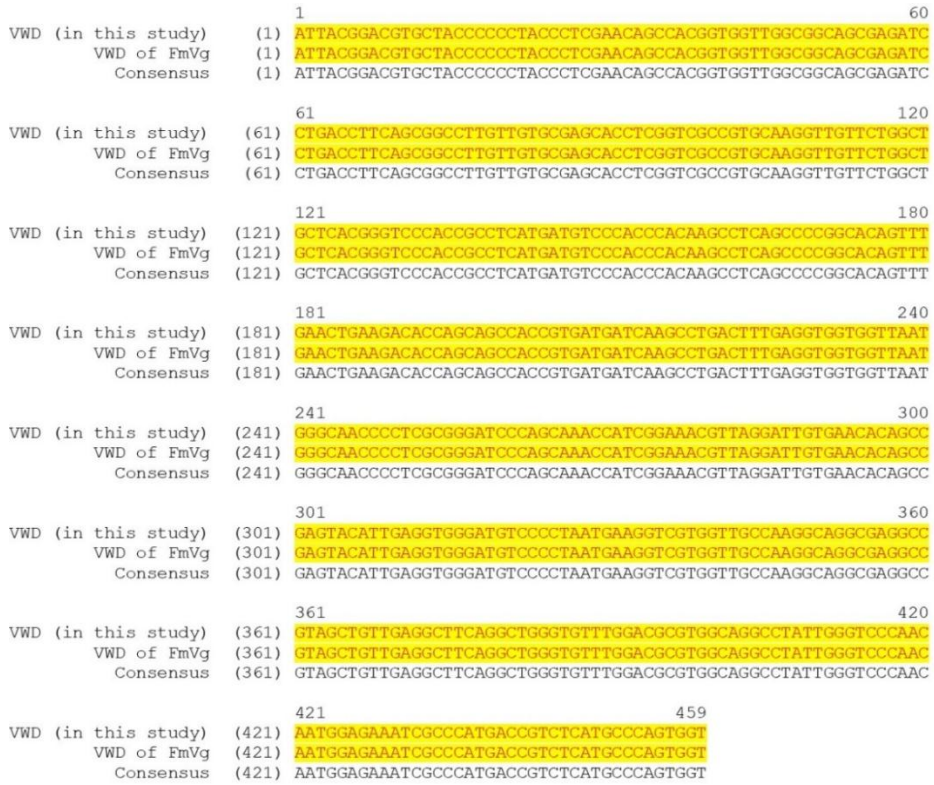


**Figure 3** Nucleotide alignment between VWD isolated from hemocytes and VWD of FmVg cloned from hepatopancreas.

Nucleotide sequences of VWD from hepatopancreas and hemocytes were aligned by Vector NTI Advance® software. Identical nucleotide was shaded as a yellow color. The number at the left displayed the order of nucleotide sequence.

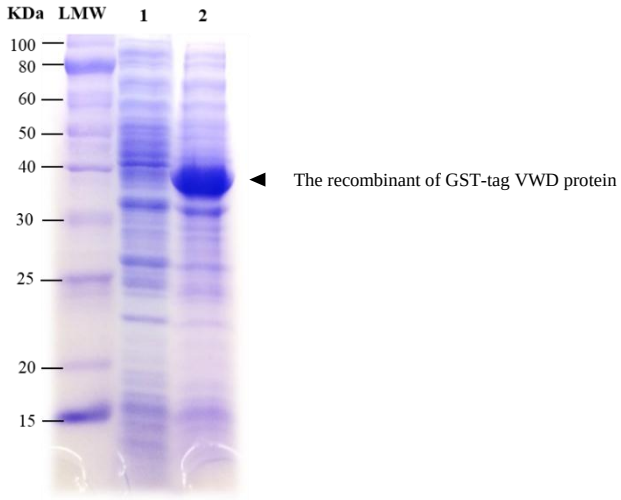


**Figure 4** The total protein expressed by *E. coli* BL21 Star™ (DE3) containing DNA of D3VWD.

The pattern of the total protein which expressions were analyzed by 12% SDS-PAGE staining with coomassie brilliant blue. Lane LMW: Low molecular weight makers, Lane1: The total protein of *E. coli* containing DNA of VWD without IPTG induction Lane2: The total protein of *E. coli* containing VWD with 1 mM IPTG induction for 4 hours.

## 5. Conclusion

VWD was successfully cloned from hemocytes of *F. merguiensis*. The length of cDNA sequence is 459 bp and we expressed this gene in pFN2A (GST) Flexi® vector. The recombinant GST-tag VWD protein was analyzed in 12% SDS-PAGE with molecular weight of 39,810 Da. In further experiments, VWD recombinant protein will be purified and applied in ELISA test to detect the interaction between VWD and FmLdlr. Moreover, we will clone the other two domains of Vg and operate the ELISA for detect the binding between Vg domains and FmLdlr.

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