ISBN 978-81-933894-3-0
5th International Conference on Agriculture, Ecology and Biological Engineering (AEBE-17)
Pattaya (Thailand), May 2-4, 2017

Optimization of *Penaeus monodon* Rab5 and Rab9 Protein Expression and Purification for Antibody Production

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Abstract: *White spot syndrome virus (WSSV) is a devastating virus and causes a serious problem in shrimp farming industry. It has been reported that WSSV entry into shrimp cells via a clathrin-mediated endocytosis pathway. Here, viruses are shuttled in cells in endosomal vesicles whose transport route regulated by Rab proteins. To investigate a hypothesis that Penaeus monodon Rab5 (PmRab5) and PmRab9 are involved in the entry of WSSV into shrimp cells, the study of subcellular localization between the two PmRab proteins and WSSV is required. In this work, we aim to optimize conditions for His_6-PmRab5 and PmRab9-His_6 protein expression and purification for antibody production. To optimize His_6-PmRab5 expression and purification, a pET28a vector containing His_6-PmRab5 was expressed in E.coli BL21* (DE3) strain. His_6-PmRab5 was purified with 1.5% sarcosine by Ni2+-NTA chromatography followed by cation exchange chromatography at pH 7 and pH 8 to compare the solubility of the protein. The best condition of His_6-PmRab5 purification was pH 7. His_6-PmRab5 was dialyzed twice against 1x PBS to remove salt and stored at 4 °C. To optimize PmRab9-His_6 expression and purification, PmRab9-His_6 was expressed in different E.coli strains and induced at different cell density and temperatures. For PmRab9-His_6 purification, denaturants including 1.5% sarcosine and 8 M urea were tested to solubilize the protein. PmRab9-His_6 was best expressed in E.coli Rosetta2 (DE3) strain and purified by Ni2+-NTA chromatography in the presence of 8 M urea. PmRab9-His_6 protein was dialyzed against 1x PBS with 4 M urea followed 1x PBS without urea. The storage temperature was also optimized and found that PmRab9-His_6 could be stored at -20 °C and -80 °C before immunization. This study showed that purification of Rab proteins should perform under a denaturing condition. Both proteins will be used to produce antibodies and subsequently used in an immunofluorescence assay to study subcellular localization of WSSV particles and Rab proteins.*

Keywords: *PmRab5; PmRab9; GTPase protein; chromatography; protein purification*

1. Introduction

White spot syndrome virus (WSSV) is a major viral pathogen in shrimp industry. It causes 100% mortality rate within 3-10 days [1]. There are many pathways that virus can entry cell such as macropinocytosis and clathrin-mediated endocytosis. Currently, it has been reported that WSSV entry into the cell occurs via a clathrin-mediated endocytosis pathway [2]. Transport of endosomal vesicles in this pathway is regulated by Rab proteins. Rab is a group of small GTP binding proteins that regulate membrane trafficking processes including vesicle budding, uncoating, docking and fusion through their effector proteins [3]. Early endosomal vesicle

targeting, late endosomal vesicle trafficking to lysosome, late endosomal vesicle trafficking to trans- Golgi network and vesicle recycling are regulated by Rab5, Rab7 or Rab9 and Rab11, respectively [4]. Although WSSV infection has been reported since 1990s [5], it is still unclear how the virus enters cells.

PmRab7 has been reported to bind to WSSV. A GST pull down assay shows that PmRab7 can bind with an envelope protein of WSSV called VP28. An *in vivo* neutralization assay shows that pre-incubation of recombinant PmRab7 with WSSV particles can reduce shrimps death [6]. Moreover, silencing of PmRab7 expression in WSSV infected shrimp by dsRNA against Rab7 can inhibit WSSV replication [7]. Taken together, these previous studies suggest that PmRab7 is a factor required for WSSV replication.

A previous study suggested that Rab5 might be involved in viral entry into shrimp cells. Silencing of PmRab5 by dsRNA against PmRab5 can inhibit virus entry and reduce viral replication. An immunofluorescence assay showed that PmRab5 colocalize with YHV particles. Taken together, PmRab5 is required for an early stage of yellow head virus infection [8].

To investigate a hypothesis that Rab5 and Rab9 are involved in entry of WSSV into shrimp cells, an investigation into subcellular localization of WSSV particles and Rab proteins involved in different stages of endosome transports is required. This study aims to optimize expression and purification of recombinant His_6-PmRab5 and PmRab9-His_6 to produce antigens for antibody production. These antibodies will subsequently be used in an immunofluorescence assay. Consequently, this study might help to find out effective prevention and treatments of WSSV infection.

2. Materials and Methods

2.1 Optimization of His_6-PmRab5 Expression

A pET28a vector containing the His_6-PmRab5 encoding insert (kindly provided by Dr.Chalermporn Ongvarrasopone) was transformed into an *Escherichia coli* BL21* (DE3) strain. The transformant was grown at 37 °C until cell density (OD_{600}) reached ~ 0.6 in LB medium supplemented with 100 µg/ml kanamycin. The culture was induced with 0.4 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG), grown for 4 h and harvested by centrifugation at 4,500 rpm for 5 min.

2.2 Optimization of His_6-PmRab5 Purification

Cell pellet of His_6-PmRab5 expressing *E. coli* was resuspended in 5 ml of phosphate buffer saline (PBS) pH 7.4 supplemented with 1.5% sarcosine, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 4 unit of DNase I (New England Biolabs (NEB), USA). Bacterial cell was lyzed by sonication. The lysate was centrifuged at 4 °C, 4,500 rpm for 5 min. Supernatant was collected as a crude protein. One ml of Ni^{2+}-NTA beads per 3.5 g of pellet was used to purify the recombinant protein. First, the Ni^{2+}-NTA beads were equilibrated with 4 column volume (CV) of lysis buffer (10 mM Tris buffer pH 8, 200 mM NaCl, 5 % glycerol, 2 mM β-mercaptoethanol, and 1 mM PMFS). The crude protein was incubated with the equilibrated Ni^{2+}-NTA beads at 4 °C for 1 h. The resin was loaded onto a column, washed with wash buffer (10 mM Tris buffer pH 8, 20 mM imidazole, 5% glycerol and 200 mM NaCl) for 20 CV. His_6-PmRab5 protein was eluted by elution buffer (10 mM Tris buffer pH 8, 250 mM imidazole, 5% glycerol and 200 mM NaCl) and analyzed on 12.5% SDS-PAGE with Coomassie Brilliant G-250 dye staining. The eluent from the affinity chromatography was further purified by a SP Sepharose High Performance column at two pH, namely 7 and 8, to compare solubility of the protein. The eluent from the affinity chromatography was diluted with buffer A_O (10 mM Tris buffer pH 7, without NaCl) to lower salt concentration in the sample to equal that in wash buffer (10 mM Tris buffer pH 7, 100 mM NaCl before loading the sample to the cation exchange column that had been equilibrated with the wash buffer. The cation exchange column was washed by the wash buffer and eluted by elution buffer (Tris buffer pH 7, 500 mM NaCl). The purified protein was analyzed by 12.5% SDS-PAGE and quantified by a Bradford assay. The purified His_6-PmRab5 protein was dialyzed twice against 1x PBS buffer at 4 °C. The purification was repeated with buffer pH 8.0.

2.3 Optimization of PmRab9-His_6 expression

PmRab9-His_6 was subcloned in a pET28a vector to generate a PmRab9-His_6 encoding insert. To optimize protein expression, the pET28a vector containing the PmRab9-His_6 encoding insert was transformed into *E.coli* BL21* (DE3), *E.coli* C41 (DE3) and *E.coli* Rosetta2 (DE3) strains. Each transformant was grown at 37 °C in 20 ml of LB media supplemented with 100 mg/ml kanamycin for BL21* (DE3) and C41 (DE3) and with 100 mg/ml kanamycin and 100 mg/ml chloramphenicol for *E.coli* Rosetta2 (DE3) strains. Cells were induced with 0.4 mM IPTG when cell density (OD_{600}) reached 0.4, 0.6 and 0.8. Protein was expressed at 16 °C overnight, 30 °C for 4 h and 37 °C for 4 h. The cultures were collected by centrifugation at 4 °C, 4,500 rpm for 5 min. The protein expression (PmRab9-His_6) was analyzed on SDS-PAGE compared to uninduced cells.

2.4 Optimization of PmRab9-His_6 purification

The protocol used to His_6-PmRab5 purification was applied for PmRab9-His_6 purification. However, 8 M urea was also employed as another denaturant during the protocol development. In the protocol with 8 M urea, the cell pellet of PmRab9-His_6 was lysed as described for His_6-PmRab5 protein. Then supernatant and pellet were separated by centrifugation at 4 °C, 4,500 rpm for 5min. Then 5 ml of lysis buffer (10 mM Tris buffer pH 8, 8 M urea, 200 mM NaCl, 5% glycerol, 2 mM β-mercaptoethanol, 1 mM PMFS and 1mg/ml lysozyme) was added into the pellet. The pellet was vortexed and incubated with lysis buffer at room temperature for 5 min. after that the mixture was centrifuged at 4 °C, 4,500 rpm for 5 min. The supernatant was corrected and incubated with Ni^{2+}-NTA beads at room temperature for 1 h. the bead-protein mixture was loaded into the column. The resin was washed with wash buffer (10 mM Tris buffer pH 8, 8 M urea, 20 mM imidazole, 5% glycerol and 200 mM NaCl) for 20 CV and eluted by elution buffer (10 mM Tris buffer pH 8, 8 M urea, 250 mM imidazole, 5% glycerol and 200 mM NaCl). The purified protein was analyzed on 12.5% SDS-PAGE and quantified by a Bradford assay. After purification, PmRab9-His_6 protein was dialyzed against 1x PBS buffer containing 4 M urea at 4 °C overnight and again against 1x PBS overnight.

Approximately, 300 µg of PmRab9-His_6 was aliquoted in 4 tubes. The purified protein was stored at room temperature, 4 °C, -20 °C and -80 °C to identify the best storage condition. The tubes were observed every day for 2 weeks. Tube which precipitation indicate that protein precipitate and the condition was not selected.

3. Results and Discussions

3.1 His_6-PmRab5 was overexpressed in *E.coli* BL21* (DE3) Strain and Purified under Denaturing Condition by using 1.5% sarcosine

His_6-PmRab5 was overexpressed in an *E.coli* BL21* (DE3) strain (**Error! Reference source not found.**A) and purified under a denaturing condition with 1.5% sarcosine by Ni^{2+}-NTA chromatography and cation exchange chromatography. After the affinity chromatography step, His_6-PmRab5 was eluted and analyzed on SDS-PAGE which showed a distinct band with molecular weight corresponding to the expected molecular weight of 23 kDa of His_6-PmRab5 (**Error! Reference source not found.**B). To remove high molecular weight impurities (Fig. 1: SDS-PAGE stained with Coomassie Brilliant G-250 dye showing (A) crude cell lysate of His6-PmRab5 expression in the E.coli BL21* (DE3) strain. Lane M: a pre-stained protein marker (Bio-Rad, USA), lane U: uninduced bacterial cell, lane I: induced bacterial cell. (B) fractions from His6-PmRab5 purification by (A) using Ni2+-NTA column Lane M: a pre-stained protein marker (Thermo Fischer Scientific, USA), lane 1: flow through, lane 2-5: washed fractions, lane 6-9: eluted fractions. (C and D) using cation chromatography. Lane M: a pre-stained protein marker (Thermo Fischer Scientific, USA), lane 1-4: washed fractions, lane 5-16: eluted fractions.B; a square bracket) present in the eluent from the Ni^{2+}-NTA chromatography step, fractions 6-9 were combined for purification by cation chromatography at two pHs. The solubility of His_6-PmRab5 at pH 7 is better than that at pH 8 because the pI of His_6-PmRab5 is approximately 8.29, hence the buffer at pH 8 was too close to the pI and makes the protein prone to aggregation. SDS-PAGE showed that the ion exchange column removed the impurities and improve the purity of the resulting recombinant protein (**Error! Reference source not found.**C and **Error! Reference source not found.**D). Fractions 9-16 from the cation chromatography step were collected and dialyzed against

twice 1x PBS. From a Bradford assay, approximately 4.76 mg His_6-PmRab5 was obtained from 1 L of cell culture. The purified protein was stored at 4 °C before immunization.

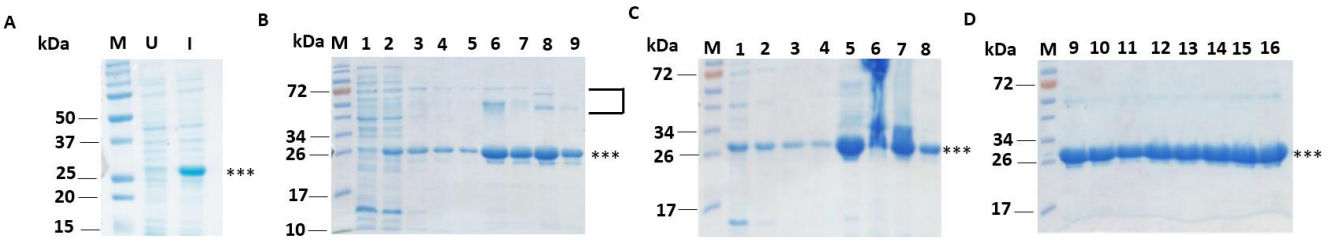

Fig. 1: SDS-PAGE stained with Coomassie Brilliant G-250 dye showing (A) crude cell lysate of His_6-PmRab5 expression in the E.coli BL21* (DE3) strain. Lane M: a pre-stained protein marker (Bio-Rad, USA), lane U: uninduced bacterial cell, lane I: induced bacterial cell. (B) fractions from His_6-PmRab5 purification by (A) using Ni^{2+}-NTA column Lane M: a pre-stained protein marker (Thermo Fischer Scientific, USA), lane 1: flow through, lane 2-5: washed fractions, lane 6-9: eluted fractions. (C and D) using cation chromatography. Lane M: a pre-stained protein marker (Thermo Fischer Scientific, USA), lane 1-4: washed fractions, lane 5-16: eluted fractions.

3.2 *E.coli* Rosetta2 (DE3) strain was the Best Expression Host for PmRab9-His_6 Expression

To determine which *E.coli* strains give the highest expression of PmRab9-His_6, the recombinant protein was expressed in 3 different *E.coli* strains, namely Rosetta2 (DE3), BL21* (DE3) and C41 (DE3) (**Error! Reference source not found.**). The overexpression of PmRab9-His_6 was highest in the Rosett2 (DE3) strain at all tested temperatures (**Error! Reference source not found.**A, 2B and 2C) and comparable irrespective of cell density at induction. Some expression of PmRab9-His_6 was detected in the *E.coli* C41 (DE3) strain but the expression level was noticeably less than that in Rosett2 (DE3). In the C41 (DE3) strain, however, the lower temperature appeared to improve the expression level (**Error! Reference source not found.**D, 2E and 2F). In contrast, no expression of PmRab9-His_6 was detected in the BL21* (DE3) strain (Fig 2*:* SDS-PAGE stained with Coomassie Brilliant G-250 dye showing of PmRab9-His6 expression in E.coli Rosetta2 (DE3) (A, B and C), E.coli BL21* (DE3) (D, E and F) and E.coli C41 (DE3) (G, H and I). Lane M: a pre-stained protein marker, lane U: uninduced bacterial cell, lane I: induced bacterial cell.G, 2H and 2I).

Rosetta2 (DE3) is an *E.coli* strain with an extra set of tRNA for rare codons including AUA, AGG, AGA, CUA, CCC and GGA. This additional tRNAs facilitate expression of a heterologous protein whose coding sequence consists of different codon preference to that of *E.coli*. For PmRab9-His_6, there are 14 rare codons, Rosett2 (DE3) might enhance the expression of PmRab9-His_6.

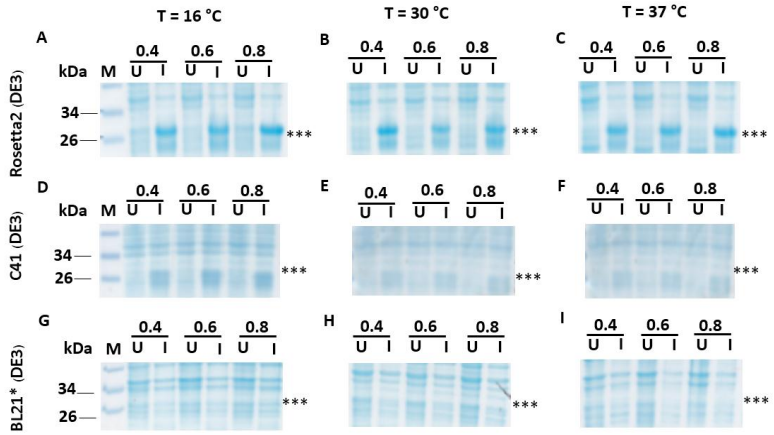

Fig 2*:* SDS-PAGE stained with Coomassie Brilliant G-250 dye showing of PmRab9-His_6 expression in E.coli Rosetta2 (DE3) (A, B and C), E.coli BL21* (DE3) (D, E and F) and E.coli C41 (DE3) (G, H and I). Lane M: a pre-stained protein marker, lane U: uninduced bacterial cell, lane I: induced bacterial cell.

3.3 PmRab9-His_6 was purified under Denaturing Condition by Using 8 M Urea

PmRab9-His_6 was overexpressed and induced at OD_{600} reached ~ 0.6 and 37 °C (**Error! Reference source not found.**A). The protein cannot be purified by Ni^{2+}-NTA chromatography with 1.5% sarcosine because it did not bind to the Ni^{2+}-NTA beads (**Error! Reference source not found.**B and 3C). Subsequently, sarcosine was replaced by a stronger denaturant, 8 M urea. In the presence of 8 M urea, PmRab9-His_6 can bind to the Ni^{2+}-NTA column. PmRab9-His_6 was eluted as describe in materials and methods and analyzed on SDS PAGE (**Error! Reference source not found.**A and 4B). SDS-PAGE showed a band with molecular weight corresponding to expected 27 kDa size of PmRab9-His_6. Then the eluted fractions 7-15 (**Error! Reference source not found.**B) were combined and quantified by the Bradford assay. Approximately 13 mg was obtained from 1 L of cell culture.

The purified protein was dialyzed against 1x PBS without urea to remove the denaturant before immunization. However, 70% of the protein aggregated. We reasoned that the aggregation was due to the abrupt change in the denaturant concentration. Accordingly, an alternative dialysis protocol was devised. The protein was initially dialyzed against 1X PBS containing 4 M urea at 4 °C overnight, followed by 1x PBS overnight. The stepwise dialysis process yielded soluble protein at approximately 9.02 mg from 1 L of *E. coli* culture.

The storage temperature was also optimized by storing protein at 4 different temperatures: room temperature, 4 °C, -20 °C and -80 °C. We found that -20 °C and -80 °C were the best temperatures because the protein precipitated at 4 °C and room temperature.

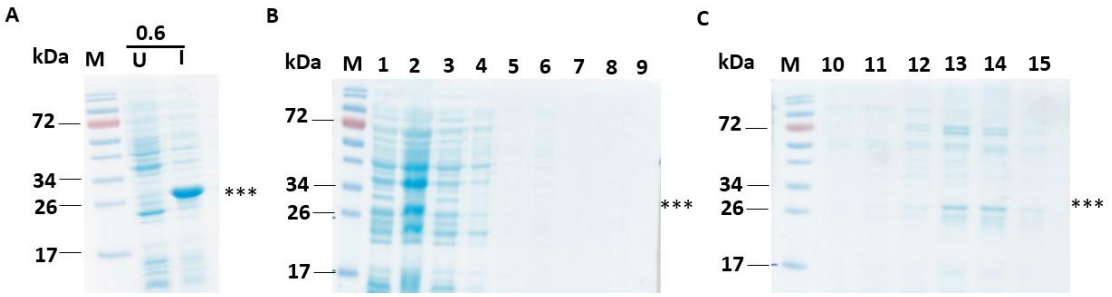

Fig. 3: SDS-PAGE stained with Coomassie Brilliant G-250 dye showing (A) crude cell lysate of PmRab9-His_6 expression in the E.coli Rosetta2 (DE3) strain. Lane M: a pre-stained protein marker lane U: uninduced bacterial cell, lane I: induced bacterial cell. (B and C) fraction from PmRab9-His_6 purified by using Ni^{2+}-NTA chromatography with 1.5% sarcosine. Lane M: a pre-stained protein marker, lane 1: flow through, lane 2-5: washed fractions, lane 6-15: eluted fractions.

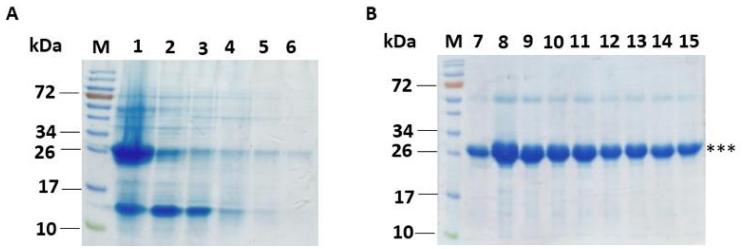

Fig. 4: A and B SDS-PAGE showing of fraction from PmRab9-His_6 purified by using Ni^{2+}-NTA chromatography with 8 M Urea. Lane M: a pre-stained protein marker, lane 1: crude protein, lane 2: flow through, lane 3-6: washed fractions, lane 7-15: eluted fractions.

4. Conclusions

The Best expression conditions for His_6-PmRab5 was in the *E. coli* BL21* (DE3) strain at 37 °C. While PmRab9-His_6 was best expressed in the *E. coli* Rosetta2 (DE3) strain at 16 °C, 30 °C and 37 °C. Both proteins were insoluble and required a denaturing condition for purification. To produce recombinant His_6-PmRab5 for

antibody production, His_6-PmRab5 was first purified by the Ni^{2+}-NTA column with 1.5% sarcosine at pH 7 followed by the cation exchange column using SP Sepharose High Performance matrix at pH 7. The total yield of His_6-PmRab5 was 4.76 mg per 1 L of cell culture. For PmRab9-His_6, we found that 8 M urea facilitated the binding of the recombinant protein to the Ni^{2+}-NTA column at pH 8. After the Ni^{2+}-NTA column, PmRab9-His_6 was sequentially dialyzed against 1x PBS with 4 M urea and 0 M urea to help protein refold. The overall 9.02 mg of PmRab9-His_6 was obtained from 1 L of cell culture. The storage temperature of His_6-PmRab5 and PmRab9-His_6 are 4 °C and -20 °C, respectively (TABLE I*: Summary of Optimization of His6-PmRab5 and PmRab9-His6 expression* and

TABLE II : Summary of Optimization of His6-PmRab5 and PmRab9-His6 purification). From this study, the Rab proteins were expressed as insoluble proteins. We suggest that it would be better to purify Rab proteins with a denaturant to get high yield of proteins.

TABLE I: Summary of Optimization of His_6-PmRab5 and PmRab9-His_6 expression

Proteins	***E. coli* Expression hosts**								
	BL21 * (DE3)			**C41 (DE3)**			**Rosetta2 (DE3)**		
	16 °C	**30** °C	**37** °C	**16** °C	**30** °C	**37** °C	**16** °C	**30** °C	**37** °C
His_6-PmRab5	x	x	+++	x	x	x	x	x	x
PmRab9-His_6	-	-	-	+	-	-	+++	+++	+++

-, bad +, poor ++, good +++, excellent x, not tried

TABLE II : Summary of Optimization of His_6-PmRab5 and PmRab9-His_6 purification

Proteins	**Denaturants**		**Solubility of proteins**		**Storage temperatures**			
	1.5% sarcosine	**8 M urea**	**pH 7**	**pH 8**	**Room temp.**	**4 °C**	**-20 °C**	**-80 °C**
His_6-PmRab5	++	x	+++	+	x	+++	x	x
PmRab9-His_6	-	+++	x	+++	-	-	+++	+++

-, bad +, poor ++, good +++, excellent x, not tried

5. Acknowledgements

I would like to thank Assoc. Prof. Dr. Chalermporn Ongvarrasopone for kindly providing the recombinant His_6-PmRab5 plasmid. This work is supported by scholarship from Faculty of Graduate studies (185781 and 254640), Mahidol University.

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