CHAPTER 5

Protein expression and analysis

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5.1 Introduction

This chapter describes the expression and partial purification of ArgRWT- and ArgRNV-biotinylated peptide fusion protein. SDS-PAGE followed by Western blotting analysis was carried out to determine the presence of the fusion proteins expressed either by *argRWT* or *argRNV* fusion genes.

5.2 Small-scale expression of ArgRWT-biotinylated peptide fusion protein

Small-scale expression showed that recombinant plasmid pKAR100 produced a protein with the size approximately 30kDa as shown by SDS-PAGE analysis (Figure 5.1). The presence of the ArgRWT-biotinylated peptide fusion protein was further analyzed using Western blotting analysis. DS956 strains were transformed separately with plasmids pKAR100 (expressing ArgRWT-biotinylated peptide fusion protein), pXa-Control Vector, and PinPoint™ Xa-3. DS956 was also used as control. The transferred protein was visualized with Ponceau-S red solution (Figure 5.2 Panel A) followed by chromogenic detection (Figure 5.2 Panel B). In lanes 1-6 (Panels A and B), a strong band migrating approximately at 30 kDa suggest that plasmid pKAR100 expressed an ArgRWT fusion protein because the monomer size of ArgRWT is 16.5 kDa (Lim *et al.*, 1987) and the biotinylated peptide is 13 kDa. The presence of several bands in the lanes containing the fusion protein may be due to degradation of the recombinant protein by cellular proteases. Strains of *E. coli* normally produce a single

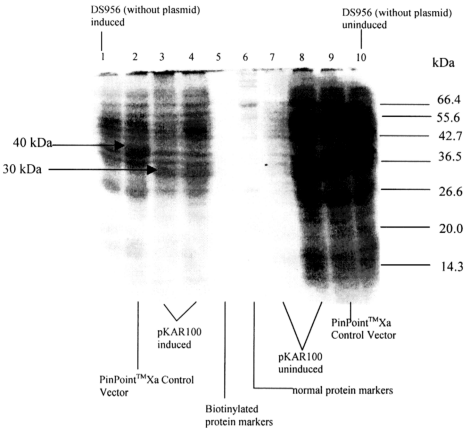

Figure 5.1. SDS-PAGE analysis of small-scale expression of ArgRWT-biotinylated peptide fusion protein. DS956 harbouring pKAR100 was induced with 1 mM IPTG. Lanes 3 and 4: Showed the expression of ArgRWT-biotinylated peptide fusion protein with the expected size (30 kDa) as indicated. Lane 8: a 30 kDa ArgRWT-biotinylated peptide fusion protein was also detected in uninduced strain DS956 (pKAR100). Lanes 2 and 9: PinPoint™ Xa Control Vector expressed a 40 kDa CAT (chloramphenicol acetyltransferase) protein. Note that DS956 without plasmid pKAR100 did not expressed a 30 kDa fusion protein (Lane 1). Lane 6: protein size markers (molecular weight of protein size markers are indicated on the right). Lane 5: biotinylated protein size markers (not detected).

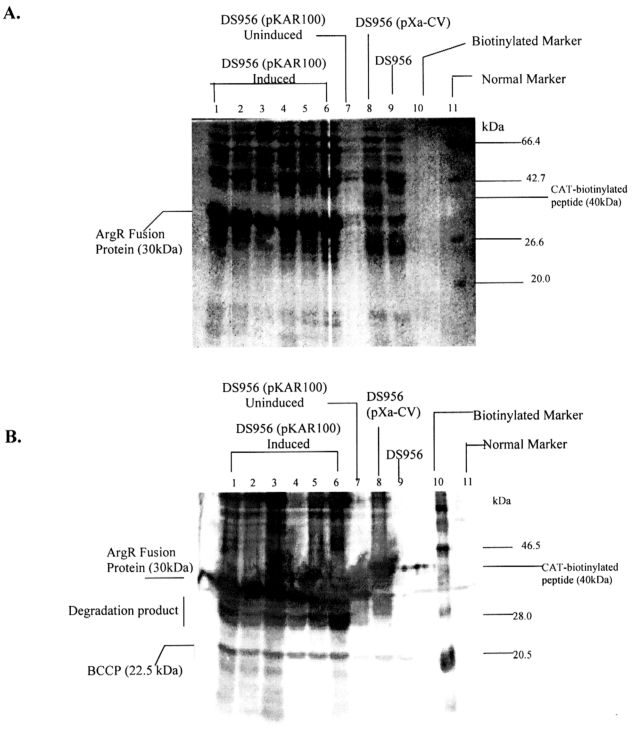

Figure 5.2: Expression of ArgRWT Fusion Protein In *E. coli* DS956. Total cellular proteins were subjected to electrophoresis on a 10% SDS-polyacrylamide gel and transferred by electroblotting onto a PVDF membrane. **Panel A:** Ponceau S-stained protein blot. The ArgR-biotinylated peptide fusion protein is approximately 30 kDa. **Panel B:** Localization of biotinylated proteins using streptavidin-alkaline phosphates conjugates and NBT/BCIP. The position and mass (kDa) of molecular weight markers are shown on the right.

endogenous 22.5 kDa biotinylated peptide protein, BCCP (Biotin Carboxyl Carrier Protein; Figure 5.2 Panel B, lanes 1-9). DS956 containing the PinPoint Xa Control vector (pXa-CV) also produce a fusion protein of approximately 40 kDa (Lane 8, Panels A and B). Note that only the endogenous BCCP can be detected in cellular extracts of DS956 cells (Figure 5.2 Panel B, Lane 9).

5.3 Small-scale expression of ArgRNV-biotinylated peptide fusion protein

Figure 5.3 shows the results of Western blotting analysis of a small-scale protein expression test for plasmid pKAR200. In lane 1, a strong band migrating approximately at 30 kDa suggest that plasmid pKAR200 expressed an ArgRNV-biotinylated peptide fusion protein because the monomer size of ArgRNV is the same with ArgRWT, which is 16.5 kDa (Burke *et al.*, 1994) and the biotinylated peptide is 13 kDa. DS956 containing the PinPoint™ Xa Control Vector (pXa-CV) also produce a fusion protein of approximately 40 kDa (lane 2) (uninduced cells containing plasmids pKAR200, and pKAR100, and an induced cells containing plasmid vector Xa-3 produced the same size protein, which is a slightly higher that 14.3 kDa of biotinylated peptide (lanes 3-5). Note that DS956 (without plasmid) produces only BCCP, that is a single endogenous 22.5 kDa biotinylated peptide protein (lane 6). This result showed that plasmids pKAR100 and pKAR200 expressed the same size proteins (ArgRWT- and ArgRNV-biotinylated peptide fusion protein respectively) of approximately 30 kDa as shown in Figure 5.4. Plasmids pKAR100-1 and pKAR200-1 in which the *argRWT* gene and argRNV gene were inserted separately into *Bam*HI-

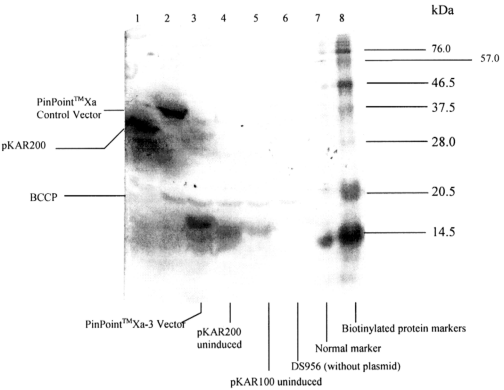

Figure 5.3. Small-scale protein expression test of ArgRNV-biotinylated peptide fusion protein. DS956 strain harbouring plasmid pKAR200 was induced with 1 mM IPTG. Lane 1: Western blotting analysis showed that the ArgRNV-biotinylated fusion protein was expressed from pKAR200 (indicated by the presence of a 30 kDa protein band). Lanes 3, 4, 5: plasmid PinPoint™ Xa-3 Vector, uninduced DS956 (pKAR200) and uninduced DS956 (pKAR100) expressed a slightly higher than 14.5 kDa protein band. It may due to a biotinylated tag protein. PinPoint™ Xa Control Vector expressed a 40 kDa chloramphenicol acetyltransferase (CAT) protein (lane 2). Note that strain DS956 without plasmid expressed only a 22.5 kDa endogenous biotinylated peptide (BCCP; lane 6). Lane 8: biotinylated size markers, the respective molecular weight are on the right (kDa). Lane 7: normal protein size markers (not detected).

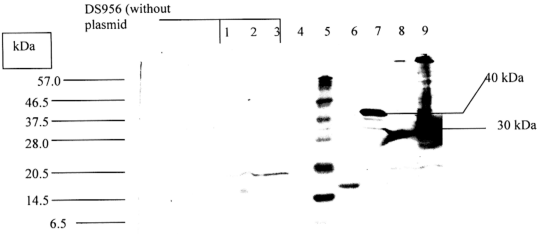

Figure 5.4. Small-scale expression of ArgRWT-biotinylated fusion protein and ArgRNV-biotinylated fusion protein in E. coli DS956. ArgRWT-biotinylated fusion protein expressed from pKAR100 and ArgRNV-biotinylated fusion protein expressed from pKAR200 have the same molecular weight with the expected size of 30 kDa (lanes 8 and 9). PinPoint™ Xa Control Vector expressed 40 kDa CAT protein (chloramphenicol acetyltransferase, lane 7. PinPoint™ Xa-3 Vector also expressed a protein which migrated slightly higher than 14.4 kDa, lane 6. Note that strain DS956 expressed only a single endogenous biotinylated protein (BCCP, lanes 1-3). Lane 5, biotinylated protein markers (the molecular weight of protein markers are on the left; kDa). Lane 4, normal protein markers are not detected.

digested PinPoint™ Xa-3 in the wrong direction were also used in this experiment. The result showed that the two recombinant plasmids, pKAR100-1 and pKAR200-1, expressed a protein band (data not shown) in the same size with the protein expressed by PinPoint™ Xa-3.

5.4 Partial purification of fusion proteins

The overall scheme for the partial purification of the fusion proteins (DS956 expressing ArgRWT- or ArgRNV-biotinylated peptide fusion protein) is summarized in Figure 5.5.

5.4.1 Expression conditions

Optimal expresion conditions were determined by varying IPTG concentrations (100 and 1 mM) and the induction condition time (3 hours, 5 hours and overnight). 20 ml bacterial cultures with the respective plasmids were firstly grown until log phase (approximately three hours, 250 rpm) and were induced with IPTG (100 μM or 1 mM). 1 ml of each culture was taken every 3 hours, 5 hours, and overnight. The results showed that 1 mM of IPTG with overnight induction time was sufficient for obtaining the maximum yield (Figure 5.6; see also Figure 5.1).

5.4.2 Fractionation of cellular proteins

In order to characterize whether the expressed fusion proteins are soluble or insoluble, induced cells were subjected to sonication and followed by centrifugation. Supernatant (soluble protein sample) and pellet (insoluble protein

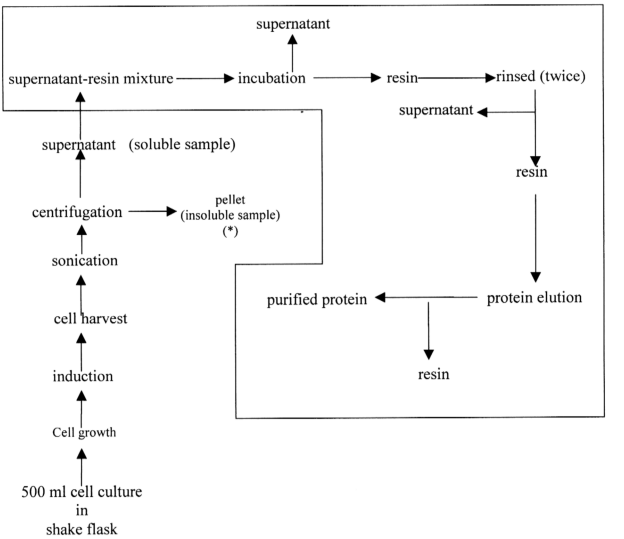

Figure 5.5. Purification scheme for the purification of ArgRWT- and ArgRNV-biotinylated peptide fusion proteins. Purification shown in this scheme followed the batch captured method (boxed). (*) The pellet (insoluble sample) that was obtained following centrifugation was resuspended in 5 ml cell lysis buffer. The protein fractions collected were analyzed using SDS-PAGE analysis.

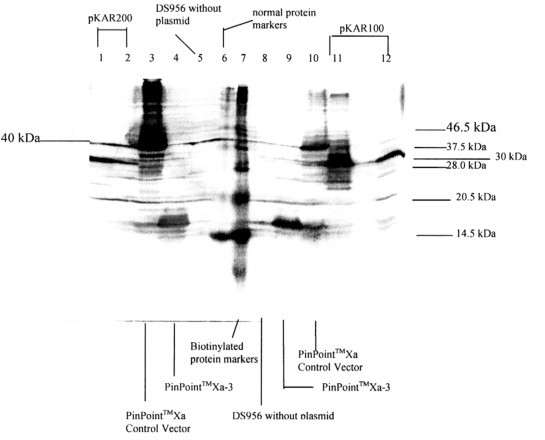

Figure 5.6. Induction time for optimal condition expression. Lanes 1 and 11: overnight induction of pKAR200 (expressing ArgRNV-biotinylated peptide fusion protein) and pKAR100 (ArgRWT-biotinylated peptide fusion protein), respectively, which resulted the maximum yield for protein expression better than the protein bands in lanes 2 and 12. Lanes 2 and 3: 3 hours induction of pKAR200 and pKAR100, respectively. Lane 7: biotinylated protein size markers (molecular weight of the markers are indicated on the right).

sample) were subjected to SDS-PAGE. The pellet was resuspended in cell lysis buffer prior to purification. The purification scheme followed was as described in Figure 5.5. Purification of proteins were carried out using Batch capture and Column capture methods.

5.4.2.1 Purification by Batch capture method

SDS-PAGE analysis showed that ArgRWT- and ArgRNV-biotinylated peptide fusion protein was well captured by the avidin resin in insoluble crude extracts (Figure 5.7). The resin-captured proteins were then eluted from the resin by cell lysis buffer containing 20 mM biotin. Note that only ArgRNV-biotinylated peptide fusion protein (in insoluble crude extract) was eluted from the resin (lane 8). SDS-PAGE analysis of ArgRNV-biotinylated peptide fusion protein fractions is shown in Figure 5.8.

5.4.2.2 Purification by Column capture method

Protein purification was also performed using Column capture method. The soluble protein sample was captured in a column containing SoftLink avidin resin. The extracts were passed through the resin several times using a vacuum pump. The resin was washed with two column-volume of buffer. The protein was eluted with cell lysis buffer containing 20 mM biotin followed by SDS-PAGE analysis. The result showed that no protein band was detected (data not shown).

Purification of ArgRWT- and ArgRNV-biotinylated peptide fusion proteins was performed in order to obtain the proteins in large quantity. A large

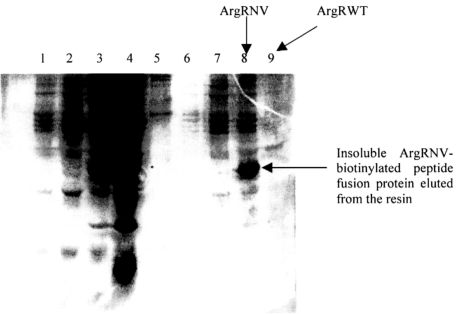

Figure 5.7. Resin capture of soluble and insoluble protein sample. Lanes 1-2: Soluble protein sample of ArgRWT- and ArgRNV-biotinylated peptide fusion protein, respectively, were not captured in the resin. Lanes 3 and 4: insoluble protein sample of ArgRWT- and ArgRNV-biotinylated peptide fusion protein, respectively were well captured in avidin resin. Lanes 6 and 7: soluble protein sample eluted from resin (ArgRWT- and ArgRNV-biotinylated peptide fusion protein, respectively). Lanes 8 and 9: insoluble protein sample eluted from resin (ArgRWT- and ArgRNV-biotinylated peptide fusion protein, respectively). Note that only insoluble ArgRNV-biotinylated peptide fusion protein was eluted from the resin with cell lysis buffer containing 20 mM biotin as indicated (lane 8). Lane 5: protein size markers (molecular weight of protein size markers are not clearly detected).

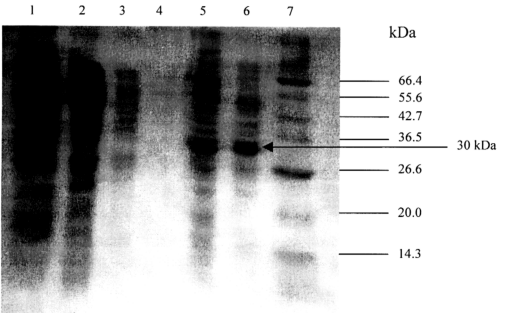

Figure 5.8. Purification fractions of insoluble ArgRNV-biotinylated peptide fusion protein. Purification scheme was as described in Figure 5.5. Lane 1: Induced DS956 (pKAR200). Lane 2: cell suspension after resin captured. Lanes 3 and 4: supernatant collected from washing steps. Lane 5: resin-captured protein. Lane 6: eluted-protein with cell lysis buffer containing 20mM biotin. Note that both resin and eluted protein contain a protein band of ArgRNV-biotinylated peptide fusion protein which migrates at approximately 30 kDa as indicated. Lane 7: protein size markers. The position and mass (kDa) of molecular weight markers are shown on the right.

quantity of the proteins is required for further studies of the proteins in conjunction with their properties in DNA-protein binding. A partial purification of both fusion proteins was carried out in order to establish the protein purification methods. During purification using Batch capture or Column capture method, I noticed that the pH of the buffer is important for the binding of the fusion protein to the SoftLink™ avidin resin (data not shown). I also noticed that the pH of the buffer changed in column. It may be due to the incomplete regeneration of the resin with the buffer column.