Purification and biochemical characterization of *Drosophila* VAP-MSP domain

A thesis submitted to

Indian Institute of Science Education and Research Pune

in partial fulfillment of the requirements for the

BS-MS Dual Degree Programme

Thesis supervisor: Dr Girish Ratnaparkhi

by

Manoj Sahu

Registration number: 20071036

2012

Indian Institute of Science Education and Research, Pune

Sai Trinity Building, Pashan, Pune India 411021

Certificate

This is to certify that this thesis entitled “Purification and biochemical characterization of *Drosophila* VAP-MSP domain'' submitted towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research Pune represents original research carried out by “Manoj Sahu, Registration No. 20071036'' at ''INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH, PUNE'', under the supervision of ''Dr. Girish Ratnaparkhi'' during the academic year 2011-2012.

Student

Manoj Sahu

Reg. No. 20071036

IISER Pune

Supervisor

Dr. Girish Ratnaparkhi

Assistant Professor,

Biology, IISER Pune

Abstract

VAP-B is an integral membrane protein, located in the endoplasmic reticulum. In humans, a P56S mutation is associated with familial forms of motor neuron disease. One effect of the mutation is the aggregation of the protein, which also pulls down wild type protein into these aggregates. The exact relationship between the mutation, the protein aggregation and motor neuron disease is not well understood.

In our laboratory we have generated a list of 132 genetic interactors of VAP-B using a reverse genetic screen in *Drosophila*. These interactors will be used to build a systems level genetic network that will be an important step in trying to understand the mechanism of Amyotrophic lateral sclerosis.

In this study, we are trying to characterize the *Drosophila* VAP major sperm protein (MSP) domain, both wild type and mutant biophysically. We are also attempting to identify novel protein interactors of VAP wild type and VAP (P56S) *in-vitro* and *in-vivo*. We have been successful in expressing both the wild type (wt) and mutant (P58S) versions of the VAP MSP in *E. coli*. Transgenic lines expressing full length, Myc tagged, VAP (wt) and VAP (P58S) in the brain have been characterized. The characterization of the purified proteins and of the interactors is ongoing. We expect our studies to complement our genetic studies and help shed light on the mechanistic basis of Amyotrophic lateral sclerosis.

Acknowledgements

I owe my deepest gratitude to Dr. Girish Ratnaparkhi for his unparallel guidance and encouragement. I am very grateful to him for sharing his knowledge of biochemistry and instilling in me the importance of methodical experimentation.

I would like to acknowledge Dr. Anuradha Ratnaparkhi for sharing her valuable suggestions and reagents without which this project would not have been possible. I am also grateful to Dr. Thomas Pucadyil for being on my thesis advisory committee and for his critical suggestion to avoid use of Sarkosyl for purification of GST-tagged proteins

I also want to thank D. Senthilkumar for sharing his knowledge on the project and giving valuable feedback and suggestion throughout my work. I am thankful to Mithila Handu and Dr. Prajwalini Mehere for their suggestion on protein purification. I would also like to acknowledge Vallari Shukla for helping me understand the *Drosophila* model system. I thank Shreeharsha TT, Ameya Mahajan, Sachin Holkar and all other biology lab members for their advice, demonstration of experimental techniques and most importantly for their friendship. .

I express my deepest gratitude to Biology department, IISER Pune for providing the opportunity to work in a common lab system.

I would also like to thank my friends H. Guhan Venkat, Neelesh Soni, P. Vivekanand Reddy, Sameer Parihar, Sarthak Parikh and Surojit Sural for their unselfish friendship, support and encouragement throughout my stay at IISER Pune.

Most importantly, I would like to thank my family for their constant encouragement throughout my undergraduate career. I especially thank my parents for their commitment to my education. Their unwavering support and encouragement helped me to go through the most challenging obstacles in life.

Table of Contents

List of figures

List of Tables

List of abbreviations

CBB	Coomassie Brilliant Blue
CDS	Circular dichroism spectroscopy
d/w	Distilled Water
DTT	Dithiothreitol
GAA	Glacial Acetic Acid
GST	Glutathione S Transferase
IPTG	Isopropyl β-D-1-ThioGalactopyranoside
M	Molar
NMR	Nuclear magnetic resonsnce
NSF	N-ethylmaleimide-sensitive factor
O/N	Overnight (12-14 hours)
PAGE	PolyAcrylamide Gel Elecrophoresis
PBS	Phosphate Buffered Saline
PMSF	PhenylMethaneSulfonylFluoride
PVDF	Polyvinylidene fluoride
rpm	Revolutions Per minute
SDS	Sodium Dodecyl Sulfate
TBS	Tris Buffered Saline

Introduction

Amyotrophic Lateral Sclerosis (ALS) also known as Lou Gehrig's disease is a late onset neurodegenerative disease affecting motor neurons. Jean-Marie Charcot and Alexix Joffroy first described it in 1869. The mean age of onset is 55-60 years. This disease causes death of motor neurons in lower brain stem and upper motor cortex. The voluntary muscle action is progressively affected leading to paralysis and eventually death due to respiratory failure (Naganska and Matyja, 2011). About 10% of cases of ALS are due to known genetic defects; while in other sporadic cases the cause is unknown. Since the mechanism of this disease is not yet understood, there is no known cure for ALS. A drug called riluzole is used for treatment, which does not cure the disease but slows down the progression of the disease (Lacomblez et al., 1996). The pathology of the disease shows that there is accumulation of proteinaceous inclusion bodies in the cell body and, which leads to death of neurons (Bruijn et al., 2004).

Genetic relation of ALS

So far more than 15 familial ALS associated gene loci have been identified (**Table 1**). Of these, mutation in Superoxide Dismutase1 (SOD1) is the best studied. SOD converts superoxide into oxygen and hydrogen peroxide (Ferraiuolo et al., 2011). SOD1 null mice do not develop motor neuron disease (Reaume et al., 1996) and removal of the normal SOD1 genes in mice that develop motor neuron disease from expressing a dismutase inactive mutant (SOD1G85R) does not affect onset or survival (Bruijn et al., 1998). This established that loss of SOD1 function is not the reason for disease rather it is the gain of toxic function.

Recently, hVAP-B present at locus 20q13.3 was identified in three forms of familial motor neuron disease: amyotrophic lateral sclerosis (ALS)-8, spinal muscular atrophy (SMA) and rapid progressing ALS (Nishimura et al., 2004).

Table 1: List of genes associated with ALS. Modified from (Ferraiuolo et al., 2011)

Type	locus	Gene	Onset/inheritance	Reference
Oxidative stress				
ALS1	21q22	Superoxide dismutase 1 (SOD1)	Adult/AD	Rosen(1993)
RNA processing				
ALS4	9q34	Senataxin (SETX)	Juvenile/AD	Chen et al. (2004)
ALS6	16p11.2	Fused in sarcoma (FUS)	Adult/AD	Kwiatkowski et al. (2009)
ALS9	14q11.2	Angiogenin (ANG)	Adult/AD	Greenway et al. (2006)
ALS10	1p36.2	TAR DNA-binding protein (TARDBP)	Adult/AD	Sreedharanet al. (2008)
Endosomal trafficking and cell signaling				
ALS2	2q33	Alsin (ALS2)	Juvenile/AR	Yang et al. (2001)
ALS11	6q21	Polyphosphoinositidephosphatase	Adult/AD	Chow et al. (2009)
ALS8	20q13.3	Vesicle-associated membrane protein-associated protein B (VAPB)	Adult/AD	Nishimura et al. (2004)
ALS12	10p13	Optineurin (OPTN)	Adult/AD and AR	Maruyama et al. (2010)
Glutamate excitotoxicity				
ND	12q24	d-amino acid oxidase (DAO)	Adult/AD	Mitchell et al. (2010)
Ubiquitin/protein degradation				
ND	9p13–p12	Valosin-containing protein (VCP)	Adult/AD	Johnson et al. (2010)
ALSX	Xp11	Ubiquilin 2 (UBQLN2)	Adult/X-linked	Deng et al. (2011)
Cytoskeleton				
ALS–dementia–PD	17q21	Microtubule-associated protein tau (MAPT)	Adult/AD	Hutton et al. (1998)
Other genes				
ALS5	15q15–q21	Spatacsin (SPG11)	Juvenile/AR	Orlacchioet al. (2010)
ALS–FTD	9p13.3	σ Non-opioid receptor 1 (SIGMAR1)	Adult/AD Juvenile/AR	Lutyet al. (2010) Al-Saifet al. (2011)
ALS–FTD	9q21–q22	Chromosome 9 open reading frame 72 (C9ORF72)	Adult/AD	Hosleret al. (2000) Renton et al. (2011) De Jesus-Hernandez et al. (2011)
Unknown genes				
ALS3	18q21	Unknown	Adult/AD	Hand et al. (2002)
ALS7	20ptel–p13	Unknown	Adult/AD	Sapp et al. (2003)
Abbreviations: AD, autosomaldominant; ALS, amyotrophic lateral sclerosis; AR, autosomal recessive; FTD, front temporal dementia; PD, Parkinson disease.				

The (Vesicle Associated Membrane Protein)- Associated Proteins (VAPs) are type II integral endoplasmic reticulum membrane proteins. The VAP family of proteins consists of three proteins VAP-A, VAP-B and VAP-C (a spliced isoform of VAP-B) (Nishimura et al., 1999). VAP is expressed in all the cells with varying level of expression, highest in the neurons (Chintapalli et al., 2007). VAP family of proteins are involved in a variety of cellular functions including lipid metabolism and transport, membrane trafficking, neurotransmitter exocytosis, unfolded protein response (UPR), cytoskeleton stability, etc (Lev et al., 2008).

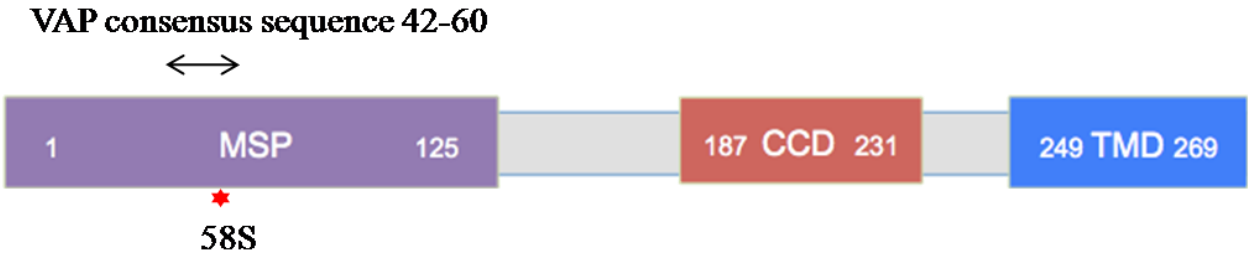

Figure 1: Domain structure of dVAP

Amino acid regions for three domains of dVAP: MSP (1-125), CCD (187-231) and TMD (249-269). VAP consensus sequence and asterisk represents position of mutated proline residue.

Structure of VAP

The VAP family of proteins is highly conserved in eukaryotes. VAP consists of three domains: an N terminal major sperm protein domain (MSP), central coiled-coil domain (CCD) and a C terminal trans-membrane domain (TMD). The MSP domain has 22% sequence identity with the major sperm protein of nematode. The CCD domain is similar to coiled-coil domain of SNARE (SNAP (soluble NSF attachment protein) receptors) proteins (Nishimura et al., 1999). The TMD domain has been shown to have a conserved GxxxG motif, which has been implicated in dimerization (Loewen and Levine, 2005).

VAP MSP domain

A highly conserved consensus sequence of 16 amino acids is present in the MSP domain. Two mis-sense mutations, which convert a conserved proline residue at position 56 into serine and threonine at 46 to isoleucine, have been identified in this conserved region of MSP domain (Chen et al., 2010; Nishimura et al., 2004). The MSP of nematode aids sperm motility by undergoing polymerization to form cytoskeletal filaments (Bottino et al., 2002). High sequence identity of VAP-MSP with nematode MSP could suggest mechanism which leads to VAP oligomerization (Lev et al., 2008).

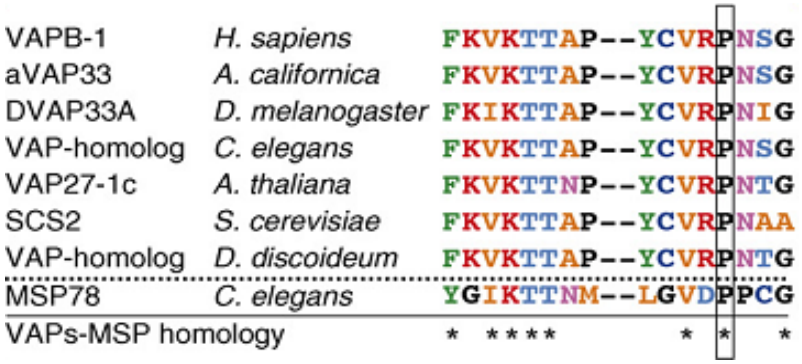

Figure 2: Comparison of the conserved MSP domain across species modified from (Lev et al., 2008)

Multiple sequence alignment of VAP consensus sequence of various species and MSP of C. elegans shows the conserved amino acids. Boxed Prolineis substituted with serine in case of ALS disease.

VAPs interact with FFAT (double phenylalanine in acidic tract) motif containing proteins. The FFAT motif consists of a conserved consensus sequence EFFDAxE (Loewen et al., 2003). This has also been established by a crystal structure of VAP where FFAT motif binds VAP in the conserved region of MSP domain (Kaiser et al., 2005). VAP homolog Scs2 in *Saccharomyces cerevisiae* regulates phospholipid metabolism via interaction with FFAT motif of *opi1*. For this function human VAP can be substituted with Scs2 but with reduced functionality (Loewen and Levine, 2005). dVAP-MSP is cleaved and acts as a ligand which binds to the Eph receptor. While P58S mutation in dVAP-MSP prevents its cleavage (Tsuda et al., 2008). This provides a whole new perspective on

the possible mechanisms that may lead to pathology of ALS. All these studies stress the important role of the MSP domain in the functioning of VAPs. Further studies on MSP domain could give us insight into the possible mechanism of ALS pathology. Therefore, it would be interesting to look at other physical interactors of VAP-MSP domain.

Drosophila VAP

Ratnaparkhi et al. have developed a *Drosophila* model for ALS using the dVAP (P58S). In the model, morphology and structure at the neuromuscular junction for both dVAP (wt) and dVAP (P58S) has been observed. The phenotype produced by neuronal expression of dVAP (P58S) resembles with the dVAP loss of function mutant. They also demonstrated that P58S mutation interferes with the BMP signaling pathway at NMJ. The overexpression of dVAP (wt) in *Drosophila* using *scabrous* driver (used for overexpression in eye and bristle) leads to decrease in number of thoracic bristle. When they co-expressed dVAP (wt) and dVAP (P58S), the dVAP (P58S) suppressed the phenotype given by dVAP (wt) overexpression showing that the mutation acts in a dominant negative manner. N-terminal hemagglutinin (HA) and Myc-tagged dVAP (wt) and VAP (P58S) were overexpressed in neurons using *elav*-GAL4 driver shows . dVAP (wt) was uniform distribution while mutant shows aggregates. They co-expressed both dVAP (wt) and (P58S) confirmed that P58S mutant VAP was recruiting wild type VAP into aggregates. (Ratnaparkhi et al., 2008). Another study done on hVAP and dVAP functionality showed that hVAP can be functionally interchangeable with dVAP. Transgenic expression of hVAP in drosophila gives similar phenotypes (locomotion defects, aggregate formation and neuronal death) as in humans, which are hallmark of the human disease (Chai et al., 2008).

Biophysical study on VAP

There have been attempt to look at the physical interactors of VAP, hVAP-MSP and hVAP-P56S were cloned and purified using nickel affinity chromatography and the purified protein bound to beads was used to pull down physically interacting form a lysate of human brain tissue. It was found out that two proteins, tubulin and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) show reduction in interaction

with hVAP-MSP hVAP-P56S as compared with hVAP-MSP (Mitne-Neto et al., 2007). This study of interactors was done using only the MSP domain not the full length proteins so; there is a chance of missing out many of the protein interactors. Biophysical characterization of hVAP-MSP (wt) and hVAP-MSP (P56S) mutant has been done. hVAP-MSP P56S was shown to always go into the inclusion bodies hence, salt free water solubility has been used and CD-spectroscopy (CDS) and NMR spectroscopy shows that it has a helical secondary structure which is different from the wild type protein. Isothermalcalorimetry (ITC) and CDS studies has also been carried out to look at the protein stability and the folding-unfolding curve with pH. ITC was done using the binding property of hVAP-MSP with FFAT motif containing Nir2 peptide (Shi et al., 2010).

Goals of the project

Broadly, the project is aimed at understanding the molecular mechanism of pathogenesis of ALS. For this a *Drosophila* model of ALS has been developed with UAS lines containing HA and Myc tagged dVAP (Ratnaparkhi et al., 2008). One part of project aims to identify the genetic interactors of VAP. The phenotypic effect of overexpression of VAP (wt) in sensory organ precursors (SOP) and knockdown of a gene using RNAi line against this background is being screened at genome level for identification of genetic interactors of VAP (Senthilkumar D, unpublished). And other part of project is *in-vitro* characterization of VAP.

My project has two components, first to get purified and soluble dVAP-MSP, perform its functional assay and biophysical characterization. Second, to perform *in-vivo* protein pull-down using VAP over-expressed in *Drosophila* neurons and find differences in the interacting proteins of both dVAP wild type and dVAP-P58S.

Materials and methods

Transformation of plasmid into bacteria

An aliquot containing 50µl competent cells (*E.Coli,* DH5α or BL21DE3) was thawed on ice for 2-5 min. ~50 ng of plasmid DNA was added to the cells, incubated on ice for 30 min. The cells were then given a heat shock at 42°C for 90 sec and immediately plunged into ice for 2 min. 1ml of LB was added to the heat shocked cells and incubated at 37°C for 1-2 hr with shaking. 100-200µl of culture was spread on LB-agar plate containing 100µg/ml ampicillin and incubated at 37°C O/N.

Protein expression

Auto-induction

A transformed bacterial colony was used to inoculate 10 ml non-inducing media containing 100µg/ml of ampicillin. This culture was grown at 37°C O/N with shaking and was then used to inoculate the inducing media, containing 100 µg/ml of ampicillin, in 1:100 (v/v) ratio and grown at 18°C for 20-24hr (Studier, 2005). Details of the composition of the inducing/non-inducing medium are in the appendix.

IPTG-induction

A transformed bacterial colony was used to inoculate 10 ml Luria broth (LB) containing 100µg/ml ampicillin. This culture was grown O/N with shaking at 37°C. Next day, the saturated culture was used to inoculate LB containing 100µg/ml ampicillin for overexpression either in 1:100 (v/v) or 1:50 (v/v) ratio. This culture was then grown at 37°C to an OD_{600} of 0.5-0.7. Protein overexpression was induced with 0.1 mM to 1.0 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and grown for an additional 4 hr - 14 hr at desired temperature for overexpression. Subsequently, the cells were harvested by centrifugation, washed twice with TBS. The cells were either processed immediately or stored at -80 °C until further processing.

Purification of proteins

GST purification of VAP (batch purification)

The cell pellet was dissolved in bacterial lysis buffer (~4 times the pellet volume). The lysis was done either by A) Sonication using Sonics Vibra-Cell VCX 130 using following program: Amplitude: 60%, Pulse: 30 sec ON and 30 sec OFF and Time: 5-10 min or B) Bioruption (time: 30 min, pulse: 15 sec ON and 15 sec OFF), till the solution cleared, indicating successful lysis. The sonicated lysate was centrifuged at high speed (>12,000 g) at 4 ºC to separate the soluble and insoluble fraction of proteins. Soluble fraction was then incubated with Glutathione Sepharose™ 4B (GE Healthcare) beads, which would separate the GST fusion proteins based on the principles of affinity purification. The bound and unbound samples were analyzed by running a 12% or 15% SDS-PAGE and stained using CBB-R250. Silver staining was also performed for higher sensitivity of detection. Elution of the bound protein was attempted using reduced glutathione (Sigma-Aldrich) using a variety of conditions (concentration of reduced glutathione and DTT, pH and ionic strength of elution buffer). Cleavage of GST-tag from the recombinant protein was done using Thrombin (Himedia). One unit of thrombin was used per 100µg of recombinant protein. Elution was also attempted using TBS, PBS and Glycine buffers.

Cloning, overexpression and purification of HIS_6-tagged VAP-MSP

The dVAP-MSP domain, both wild type and mutant, were amplified from full length dVAP (Procured from Dr. Anuradha Ratnaparkhi) by PCR (**TABLE 2**). Amplified products were cloned into Novagen pET-15b (kind gift from Shreeharsha TT) at Bam HI and Nde I restriction site (New England Biolabs, Inc.) and ligation was performed using Ligation Mighty Mix (Takara Biosciences). Ligation was performed by adding digested vector and PCR products in nearly 1:3 ratio and incubated with ligase at 16°C for 16-18 hrs. Ligated product was then transformed into *E. Coli* DH5α competent cells. The clones were confirmed by colony PCR using *T7* forward primer and dVAP-MSP reverse primer and also by restriction digestion with Nde I and Bam HI. The clones were finally

confirmed by sequencing done by 1st BASE sequencing services, Malaysia and compared with original sequence using Serial Cloner. The positive clones were transformed by heat shock into *E. Coli* BL21 DE3 competent cells for over-expression. Several of the transformed colonies were then screened for over-expression at induction condition of 25°C temperature, 1mM IPTG conc., 6 hr induction time. The Denaturing conditions were tried using Urea (8M) and Guanidinium hydrochloride (6M). Buffer used: Phosphate buffer (Buffer 'A', see appendix). The purification was done using Ni-NTA superflow beads (Qiagen) by binding it at 4°C O/N in presence of 50mM Imidazole to reduce non-specific binding. The elution was done by incubating the protein bound beads with elution buffer (buffer 'A' + 350mM imidazole + 8M urea) at room temperature for 2-3 hrs. Refolding was tried with different conditions since the proteins tended to aggregate.

Table 2: Program for PCR amplification of dVAP-MSP wild type and mutant

#	*Conditions*
1	94°C, 5 min
2	94°C, 40 sec
3	53°C, 45 sec
4	72°C, 35 sec
5	Repeat 2,3 & 4; 4 times
6	94°C, 40 sec
7	58°C, 45 sec
8	72°C, 35 sec
9	Repeat 6,7 & 8; 29 times
10	72°C, 5 min
11	4°C, hold

Table 3: Reagent concentration information for PCR reactions

Reagent	*Concentration*
$MgCl_2$	2mM
10 X Buffer 'B'	1X
dNTPs	0.1 mM each
Primer Forward	1.0 µM
Primer Reverse	1.0 µM
Taq Polymerase	0.5 Unit
Template DNA	50ng-100ng
H_2O	Up to volume

All the above reagents used for PCR were from GeNei™.

Table 4: Primers

Primer	*Sequence*
VAP-MSP-HIS-FP	GTCAGCATATG**ATGAGCAAATCACTCTTTGATCTT**
VAP-MSP-HIS-RP	GCTCCAGTCGACTCA**GCACTTCAGTTTGGCGTCCATCA**

Underline is for complementary region to template.

Pull down of HA and Myc tagged dVAP (wt) and (P58S) from Drosophila

In *Drosophil*a, a system for targeted gene expression has been developed known as UAS-GAL4 system. This method of gene regulation originally identified in yeast *Saccharomyces cerevisiae* has been incorporated into *Drosophila* as a tool for targeted gene overexpression. In this system the flies are maintained as two separate stocks of UAS and GAL4. Gene of interest is under the UAS enhancer sequence and GAL4 gene is under the control of a specific promoter. This binary system has several advantages. First, transcriptional inactivity for example, if the expression of a gene is toxic to animal it would be difficult to maintain the flies, but here it is maintained in a dormant state. Another advantage is that crossing of UAS with specific GAL4 drivers gives the ability to express the gene in a variety of spatial and temporal conditions.

Since we are looking at the role VAP in ALS, *elav*-GAL4 lines were used for expression of different UAS lines. *elav*-GAL4 drives the expression of target gene in neurons (Lin and Goodman, 1994).

The following UAS lines were obtained from Dr. Anuradha Ratnaparkhi:

- UAS-HA-VAP^{wt}
- UAS-HA-VAP^{P58S}
- UAS-Myc-VAP^{wt}
- UAS-Myc-VAP^{P58S}

These lines were then crossed with elav-GAL4 and the F1 flies were checked by western blotting. For western, 10 flies were crushed in 40µl 5X-SDS loading dye diluted with TBS, heated at 95°C for 10 minutes and 20µl was loaded on a 12% SDS-polyacrylamide gel. The protein bands were then transferred to blot (PVDF membrane) using western transfer buffer at constant current of 115mA O/N. After transfer, the blot was incubated with 5% skimmed milk in TBST (TBS+0.1% Tween 20) for 1 hr at RT for blocking, incubated with primary antibodies (α-HA rabbit monoclonal, Sigma, 1:1000) and α-Myc mouse monoclonal, Thermo scientific, 1:1000) for 3hr at RT or O/N at 4°C. The blots were washed thrice with TBST for 10 min each, followed by incubation with HRP conjugated secondary antibodies (Jackson Immunoresearch) against the primary

diluted in blocking (1:10,000 dilution), for 1-2 hrs at RT. The secondary antibody was removed and the blot was washed twice with TBST for 10 min each, rinsed with TBS and stored in TBS at 4°C. For developing the blot 100µl of LuminataTM Forte (Millipore) western substrate was spread evenly on the blot and developed using the Fuji LAS4000 system.

Results and discussions

Purification of GST-VAP-MSP

The dVAP-MSP and dAP-MSP P58S cloned by D. Senthilkumar in pGEX-4T1 were transformed into *E. Coli* BL21DE3 cells for overexpression. Overexpression of the fusion proteins was tried using both auto-induction and IPTG induction (**Figure 3**). It was found that IPTG induction gave higher yield of protein as compared to auto-induction then for IPTG induction, condition for over expression was standardized to maximize amount of the fusion protein in the soluble fraction by varying conditions such as temperature (18°C, 25°C and 37°C), conc. of IPTG (0.2mM, 0.5mM and 1mM). Several cell lysis buffer compositions were tried by varying additives in the buffer: Triton X-100 (0.5%, 1.0% and 2% v/v), EDTA (1mM), DTT (1mM) and protease inhibitor (cOmplete, Mini, EDTA-free protease inhibitor tablet from Roche Applied Science and PMSF 1mM). Although these conditions did not improve the yield of soluble protein, the GST fusion proteins were bound to glutathione sepharose beads for purification. SDS-PAGE analysis of protein lysate, protein bound beads and lysate flow through; showed that most of the protein was binding to the beads (**Figure 4**). The elution of bead bound protein was tried using different concentrations of reduced Glutathione in the lysis buffer (5mM, 10mM, 20mM and 50mM). However the elution of fusion proteins was not successful. To get the protein out from beads an alternate approach was adopted which was to cleave the proteins from the GST tag using thrombin cleavage site present between the tag and proteins. Although the cleavage was successful with thrombin, the problem of eluting protein from beads still persisted; the cleaved protein remained bound to beads and did not elute (**Figure 5**). This could have been due to several reasons such as if the proteins were precipitating or they were forming aggregate in the beads along with GST tag or the proteins are getting oligomerized themselves given that the MSP of *C-elegans*is known to polymerize and VAP-MSP has high sequence identity with it. It had been shown that Sarkosyl, although being a detergent could be used along with Triton X-100 for purification of GST tagged proteins (Park et al., 2011; Tao et al., 2010).

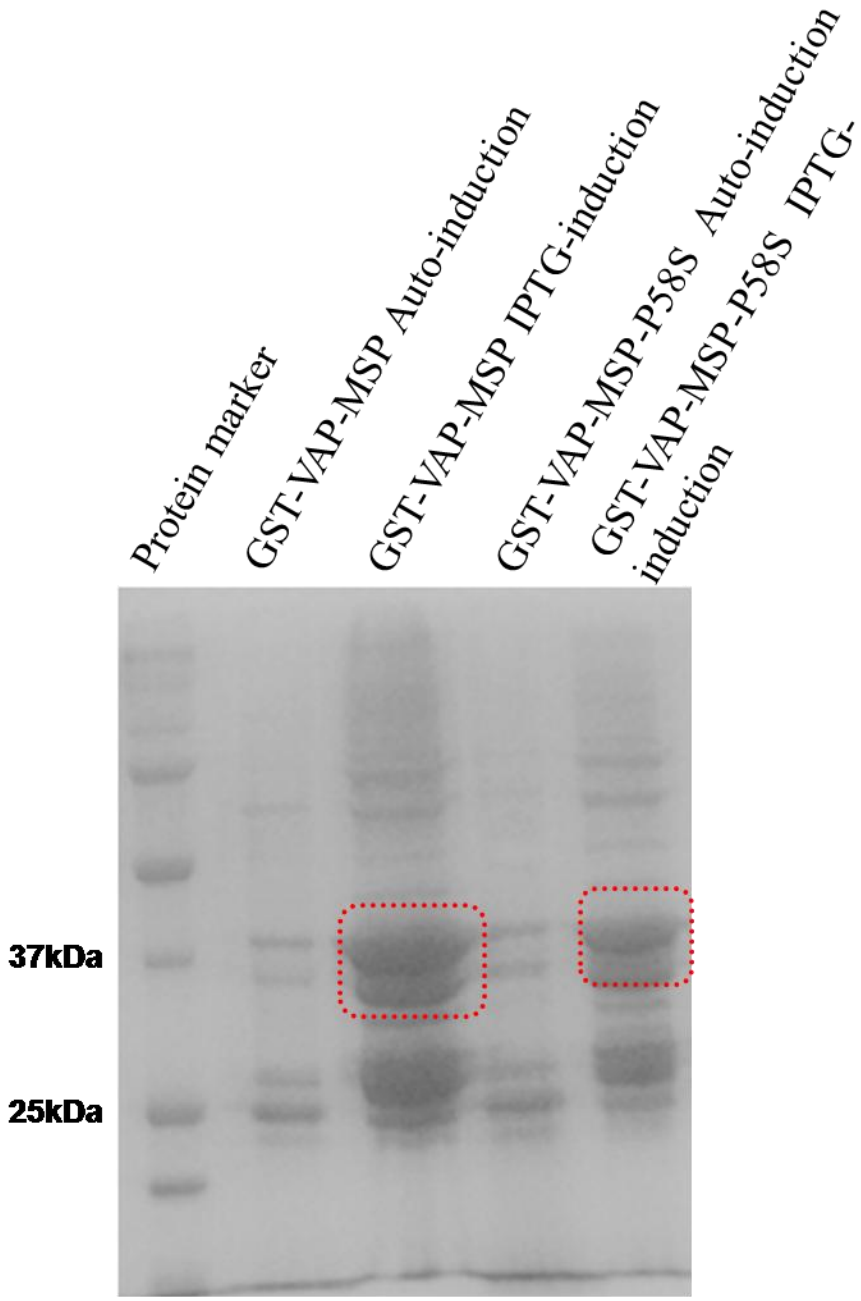

Figure 3: Comparison of GST-VAP-MSP overexpression by auto-induction and IPTG induction.

12% SDS-PAGE, stained with CBB; 1ml culture of each was used to load on the gel. This shows that auto-induction was not as efficient as IPTG-induction.

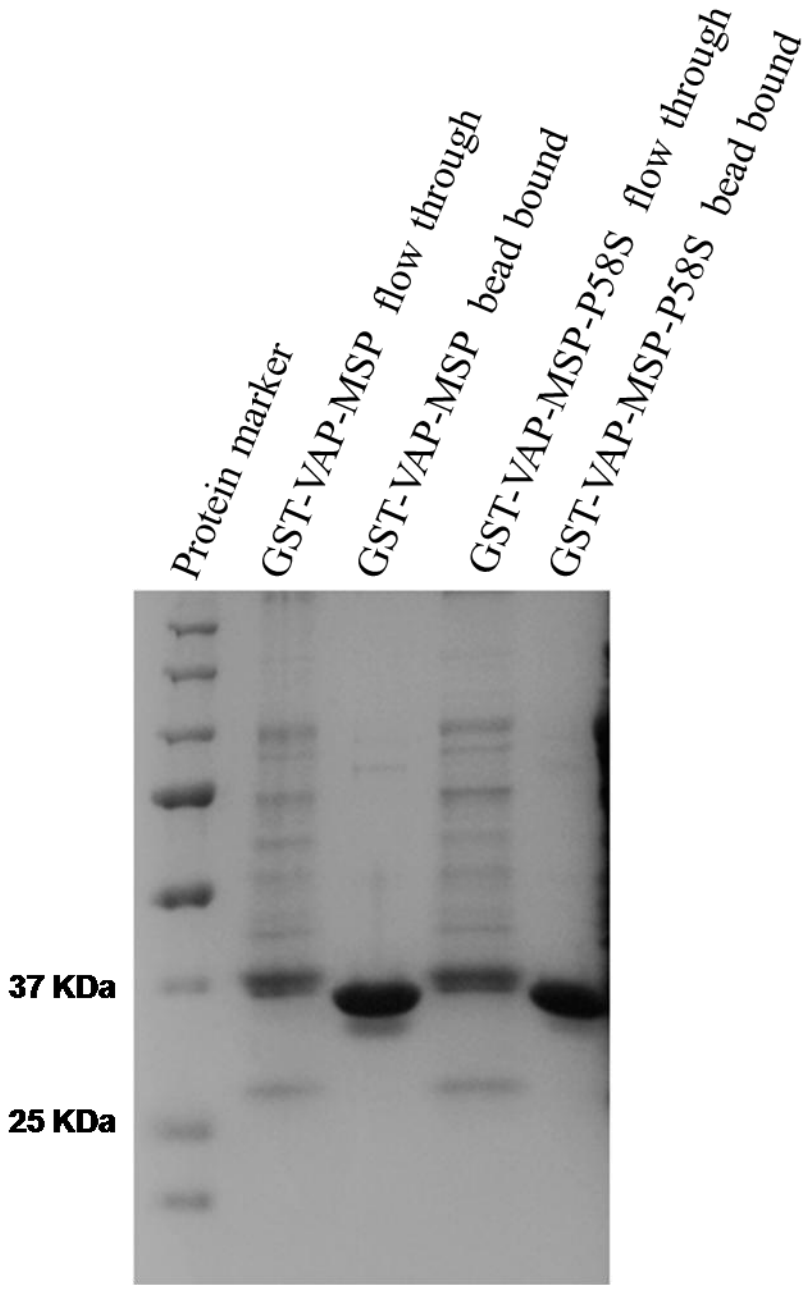

Figure 4: Purification of GST-VAP-MSP and GST-VAP-MSP-P58S

12% SDS-PAGE, stained with CBB. Binding of fusion protein to GST beads was successful and gave a clean purification. Approximately 1mg/liters culture was obtained.

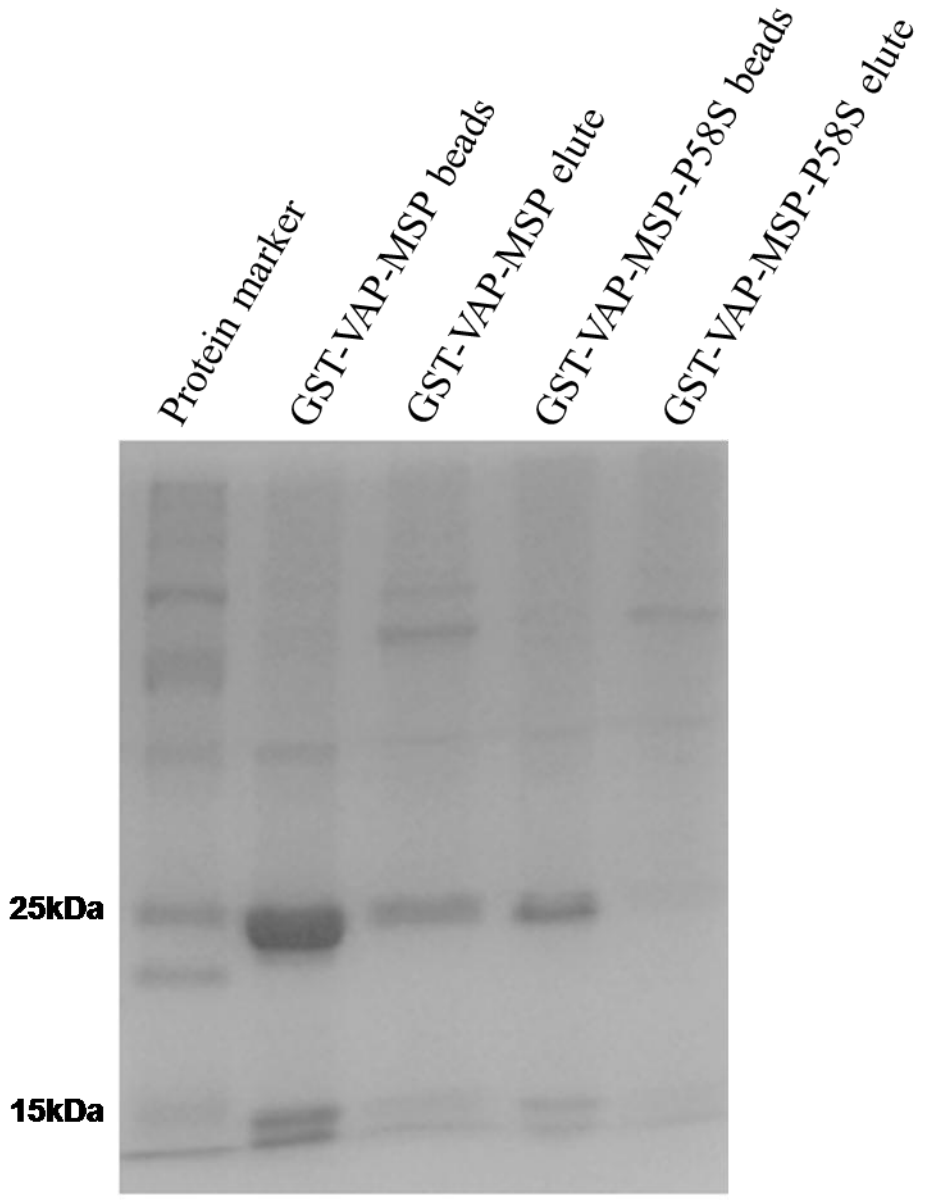

Figure 5: Elution of GST-VAP-MSP and GST-VAP-MSP-P58S by Thrombin cleavage.

12% SDS-PAGE, stained with CBB. The elution of fusion proteins from beads was not efficient, compare lane 2 & 3 and 4 & 5. Most of the cleaved protein remained bound to beads. The 25kDa fragment is of cleaved GST tag

After exploring different ratios of Sarkosyl and Triton X-100 for purification, conditions were standardized. Induction with 1mM IPTG (OD_{600} ~0.6) at 25°C for 6 hrs and cell lysis with buffer of composition 5% (w/v) Sarkosyl, 1mM DTT and 1mM PMSF in TBS gave the highest yield in the soluble fraction. The purification was done using glutathione sepharose beads and 2% (v/v) Triton X-100 was added to facilitate the binding of fusion protein to beads. However, the fusion protein could not be eluted from beads using reduced glutathione. Changing conditions, such as increasing pH (7-9), increasing salt concentration (0.1-0.2 mM NaCl) and increasing reduced glutathione concentration (10-50 mM) also failed to elute the fusion protein. Attempts to cleave the fusion protein using thrombin worked, but the cleaved products (dVAP-MSP (wt) and dVAP-MSP (P56S) remained bound to the beads. To address this problem methodical analysis of different conditions were explored. The methods included trials with ionic and non-ionic detergents, urea (up to 2M), salt (up to 0.2 M), buffers (TBS/PBS) and low pH (glycine/citric acid) (**Figure 6**). Finally, a method to successfully elute pure and cleaved protein from GST beads was standardized. If a fraction of protein was misfolded and it was preventing normal protein from coming out of beads, to check this hypothesis the time required for elution of protein was investigated supposing folded protein to easily come out. We found that at low pH all of the protein eluted very quickly, hence no definite conclusion could be reached (**Figure 7**).The eluted proteins were then renormalized by titrating with NaOH, tris and PBS buffers and checked if the protein precipitates out during the process (**Figure 8**). However, it was realized that this process of purification as a whole was not reliable since, the mechanism of Sarkosyl and its effect on the protein could not be predicted plus there was acid induced denaturation of proteins. So, it was decided to try the purification of proteins using HIS_6 tag.

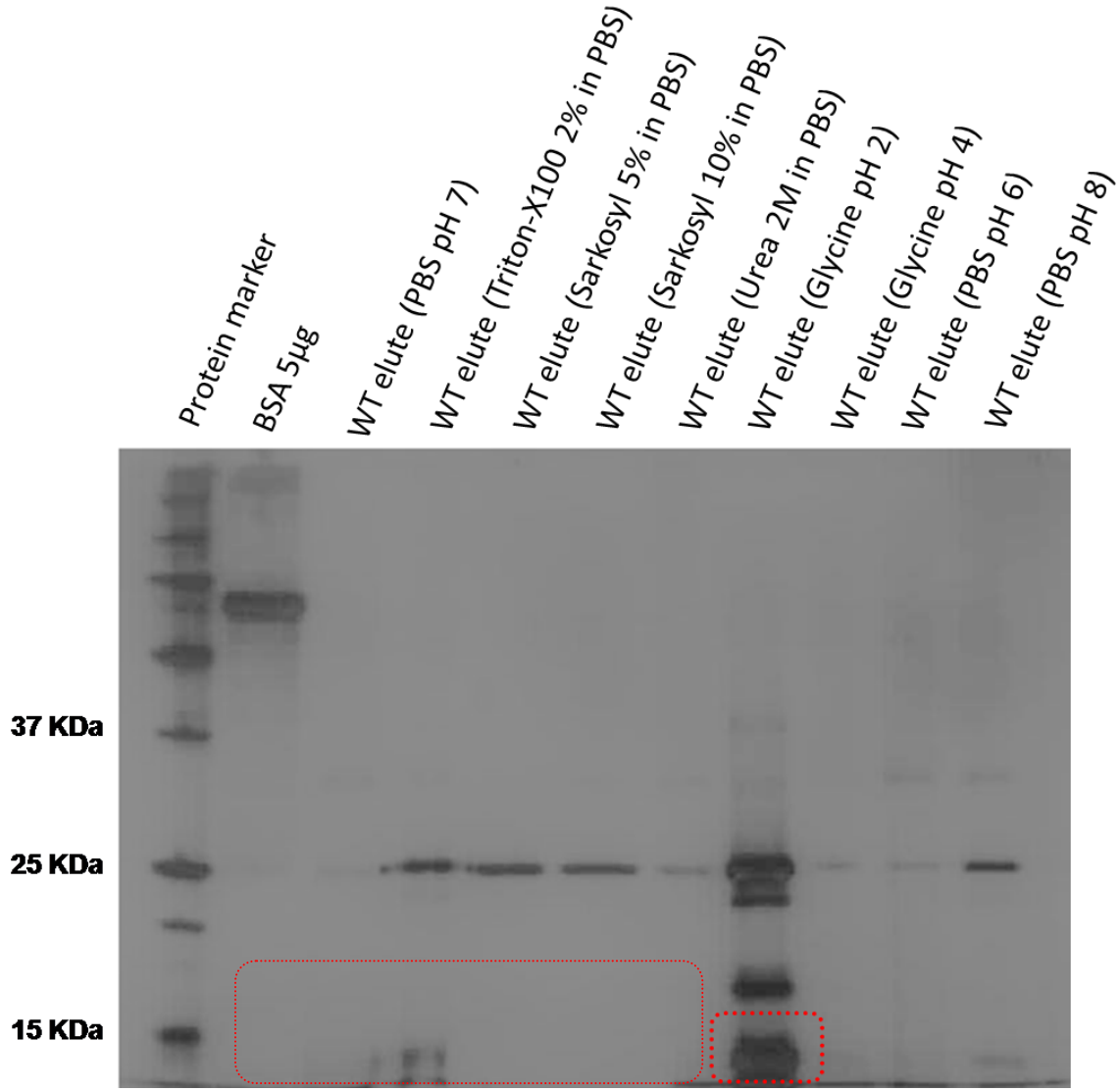

Figure 6: Elution of GST-VAP-MSP after Thrombin cleavage at different conditions

The elution of cleaved protein from the beads was tried using the above conditions. The dotted box points to the eluted protein obtained using low pH glycine buffer. With the exception of the low PH glycine buffer, in all other conditions, the cleaved protein stuck to the beads. 12% SDS-PAGE, silver stained.

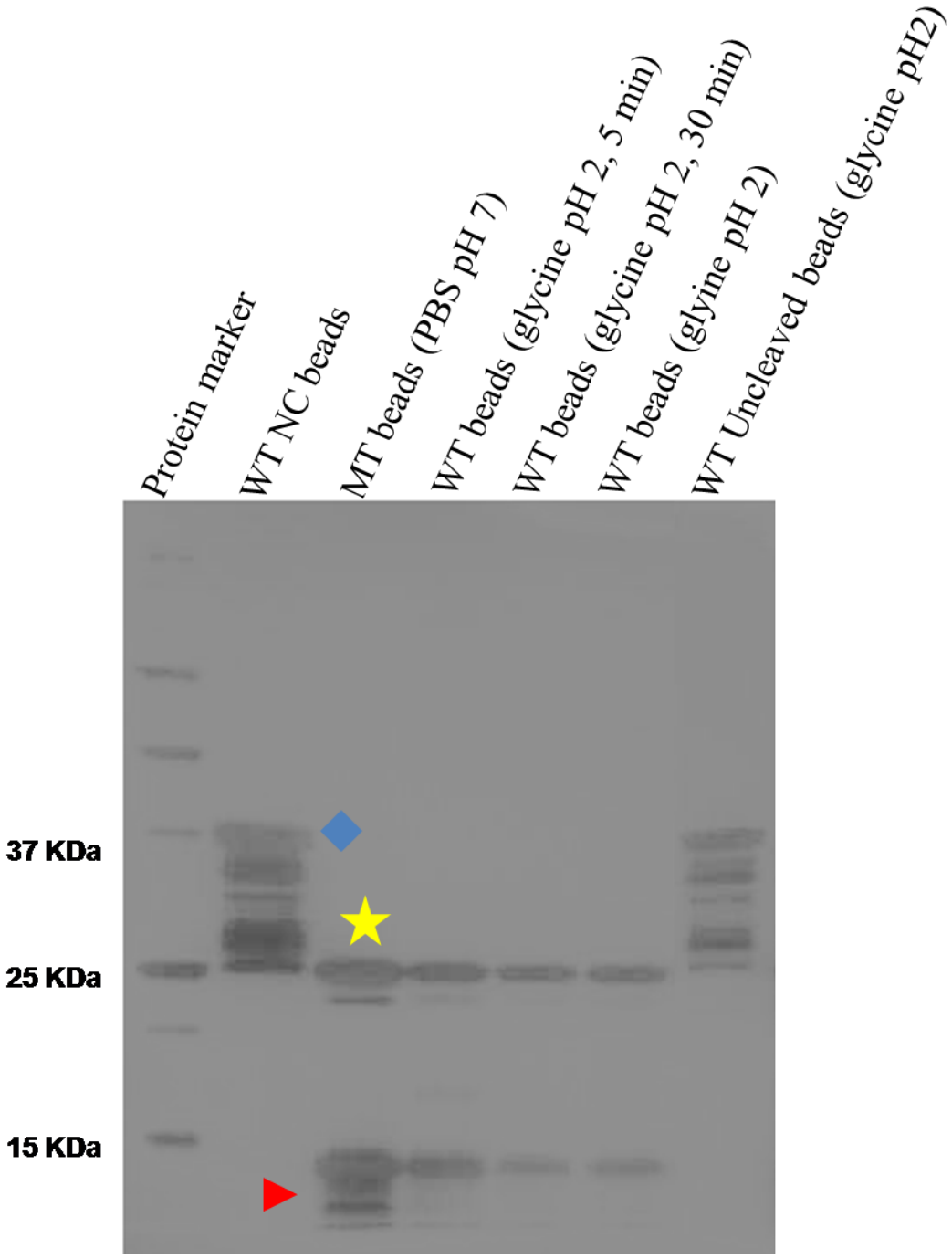

Figure 7: Time dependence of protein elution

12% SDS-PAGE, silver stained. The elution of cleaved fusion proteins from beads was not time dependent. Compare lane 4, 5 and 6. Lane 3 shows that cleaved MSP-P58S is not eluted at pH 7. Diamond points to uncleaved protein, star to GST-tag fragment and arrow-head to the MSP domain.

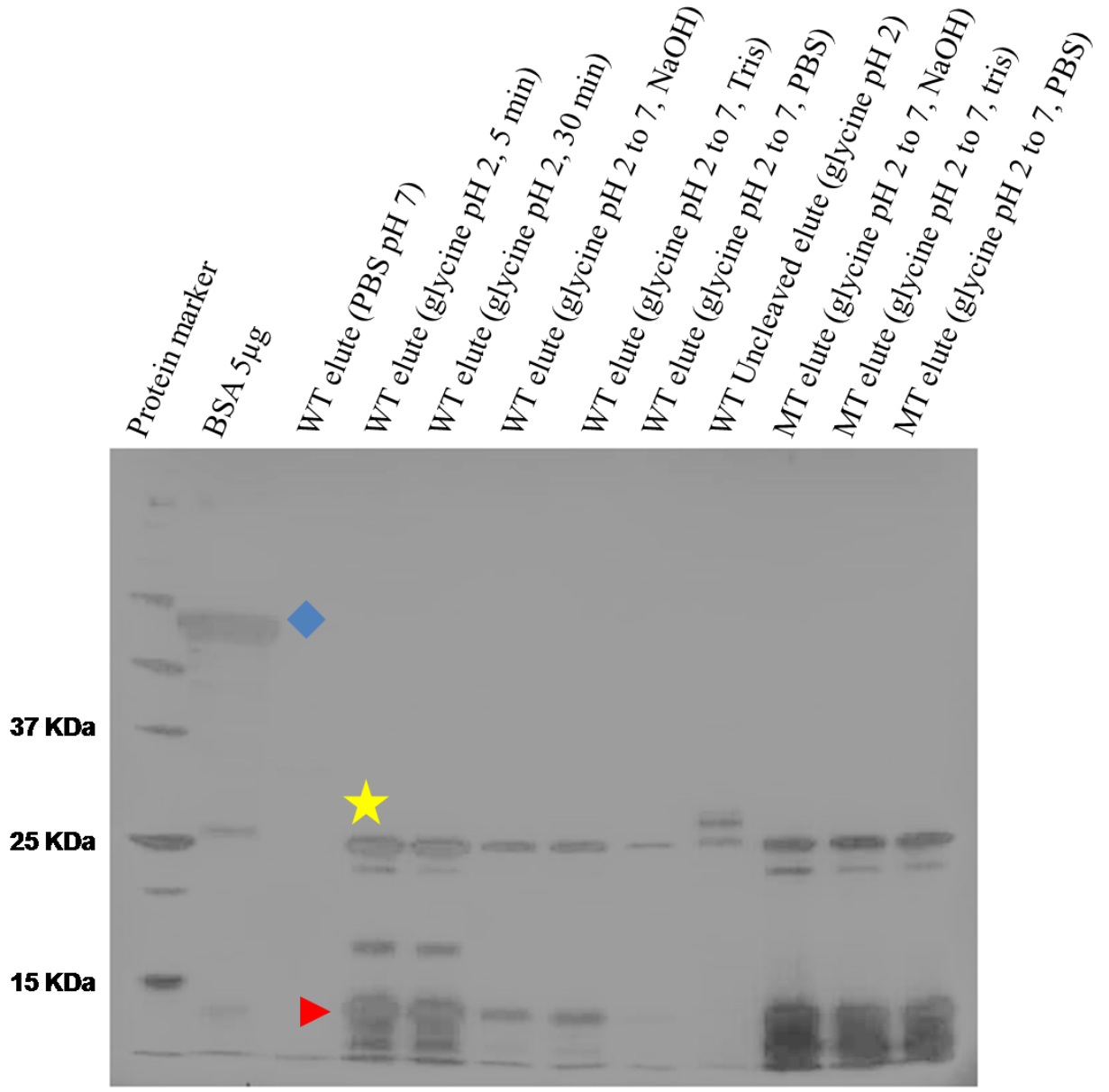

Figure 8: Renormalization of pH after elution

12% SDS-PAGE, silver stained. The eluted protein at low pH were renormalized to pH 7-8 using NaOH, Tris and PBS. Lane 3 and 9 are used as controls. The pH of the solution was renormalized and the final solution spun down to see if there was any precipitation of proteins. The supernatant after centrifuging was used to load on gel. Diamond points to BSA used as loading standard. Star points to cleaved GST tag and arrow-head points the MSP proteins.

Purification of HIS-VAP-MSP

The dVAP-MSP (wt) and dVAP-MSP (P58S) were amplified from full length sequences (provided by Dr. Anuradha Ratnaparkhi), cloned into Novagen pET-15b using Nde I and BamH I restriction sites (**Figure 9**). The clones were confirmed by PCR of the isolated plasmids using the *T7* promoter primer and reverse primer for MSP domain, to be sure about the orientation and size of the sequence (

Figure 10). The clones were reconfirmed by digestion with Bam H I and Nde I, which gave a fragment of 375bp. The clones were sequenced by 1stbase Sequencing Services, Malaysia (**Figure 11**) and the sequences were found to match the expected sequences (not shown). The confirmed clones were then transformed into *E. Coli* BL21 DE3 cells for overexpression. Two clones from each HIS-dVAP-MSP (wt) and HIS-dVAP-MSP (P58S) were checked for overexpression (**Figure 12**). Conditions were tried to get the proteins in the soluble fraction but, since it was difficult to get the proteins in the soluble fraction, finally the conditions were optimized to get maximum yield of protein and then purifying it in denaturing condition. Tris and phosphate buffers were tried with either 8 M urea or 6 M Guanidinium hydrochloride (Gu-HCl). It was found out that denaturing the proteins in 8M urea gave better yield as compared to 6M Gu-HCl (**Figure 13**). For refolding the proteins, dialysis was done with a step gradient reducing the concentration of urea by 5 folds during each spin. This method was not successful as it leads to the aggregation and precipitation of the proteins from solution when urea concentration was decreased below 3 M. The reason behind precipitation could be the sudden change in urea concentration, so a slow step gradient was tried for refolding. The dilution was done in steps of 2 fold till concentration approximately reached 3M (after which the protein was precipitating) then the dilution was slowed more up to ~1.2-1.3 folds per spin. Even slow dilution did not solve the problem of precipitation. The other reason for precipitation could be the buffer composition. There are two kinds of approaches to deal with precipitation of protein during refolding: 1) Folding enhancers – enhances protein-protein interaction and stability. 2) Aggregation suppressors – does not interfere with folding and reduces side chain interaction of folding intermediates. One additive from each category: 0.1 % (w/v) PEG-3500 and 10% (v/v) Glycerol were used to check if it would help with the refolding process. It was found that addition of

10% (v/v) glycerol improved refolding and prevented the proteins from aggregation. This is now being confirmed and standardized, to get pure and soluble protein. After getting soluble protein it can be checked for functional activity by looking at the binding with FFAT-motif containing peptides.

The problem of wild type protein going into inclusion bodies is new and different from hVAP-MSP where there was no problem in getting soluble wild type protein. Biophysical studies can be done on both wild type and mutant proteins and the difference in wild type and mutant can be compared with the results from (Shi et al., 2010).

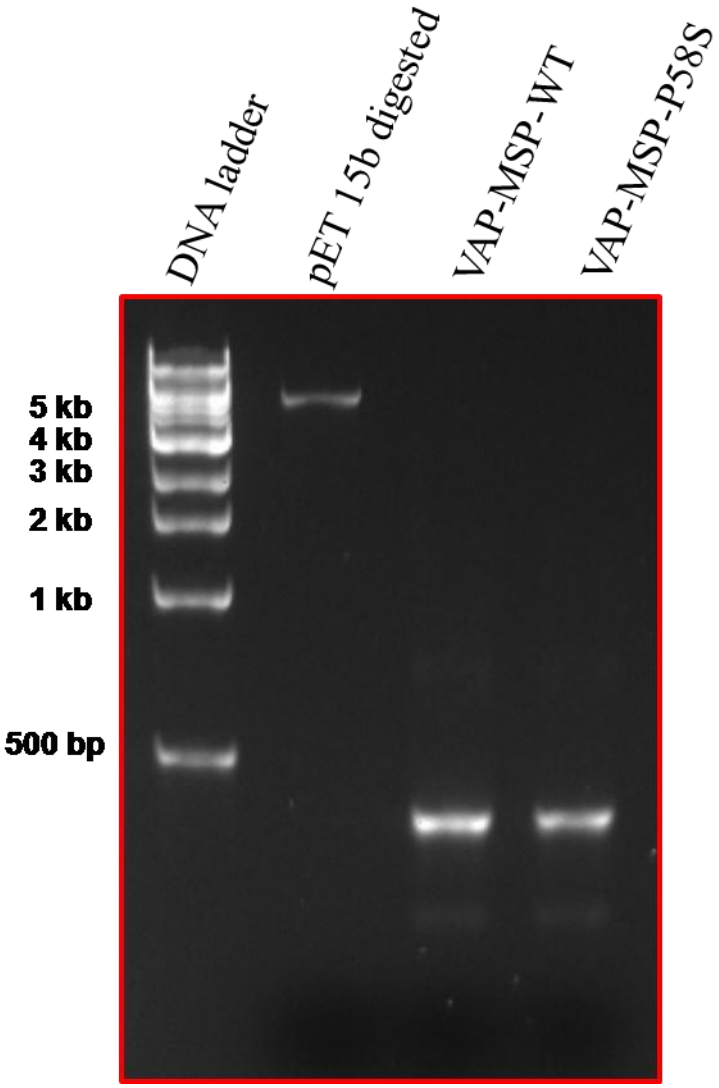

Figure 9: PCR product for dVAP-MSP wild type and mutant

Lane 2 shows pET 15b vector linearized with Bam-HI and Nde I. Lanes 3 and 4 are for the PCR amplified products used for cloning. Expected Size of dVAP-MSP was 375bp.1% Agarose gel, EtBr stained.

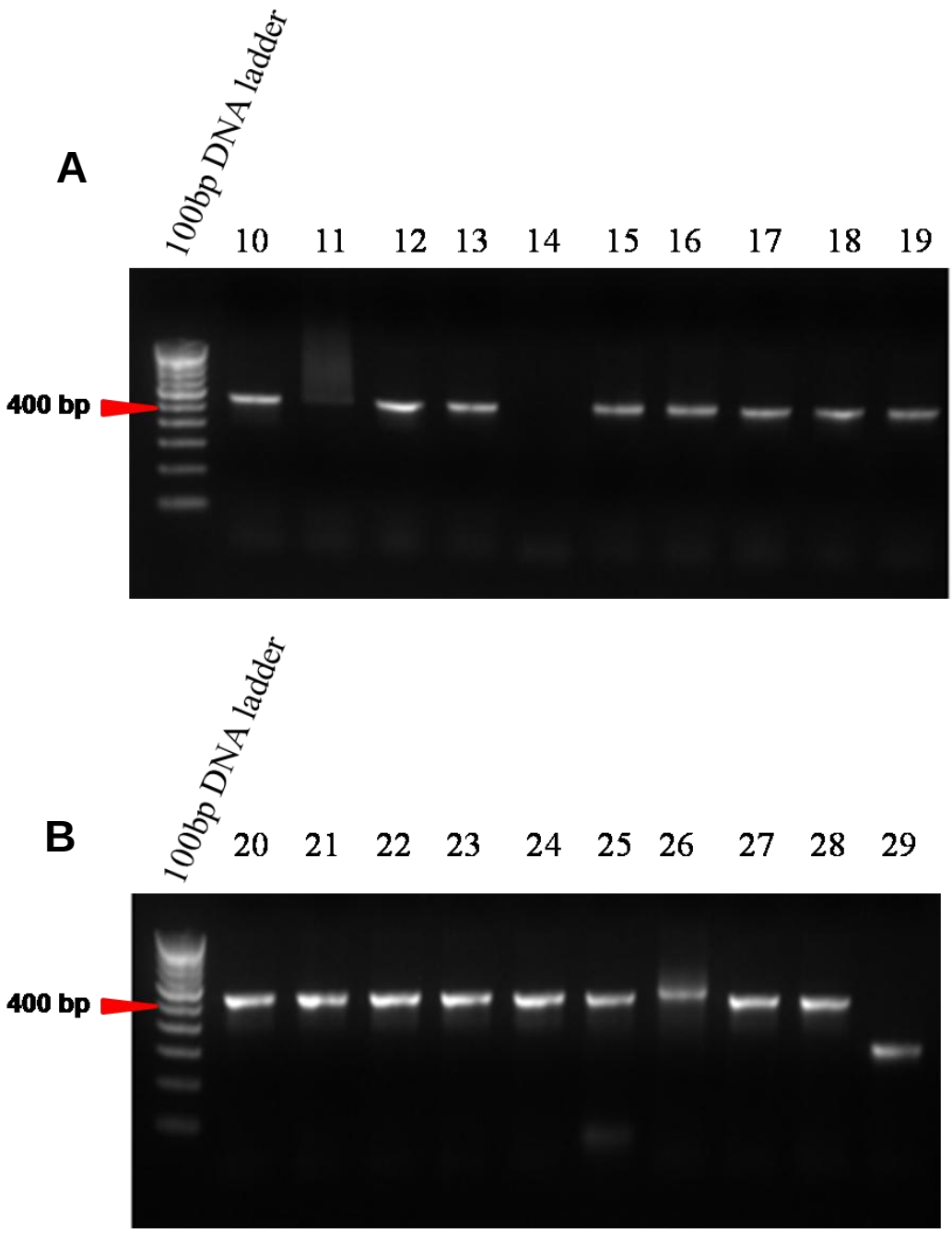

Figure 10: PCR based screening for positive clones

A. PCR product for screening of HIS-VAP-MSP wild type

B. PCR product for screening of HIS-VAP-MSP P58S

1.5% Agarose gel, EtBr stained

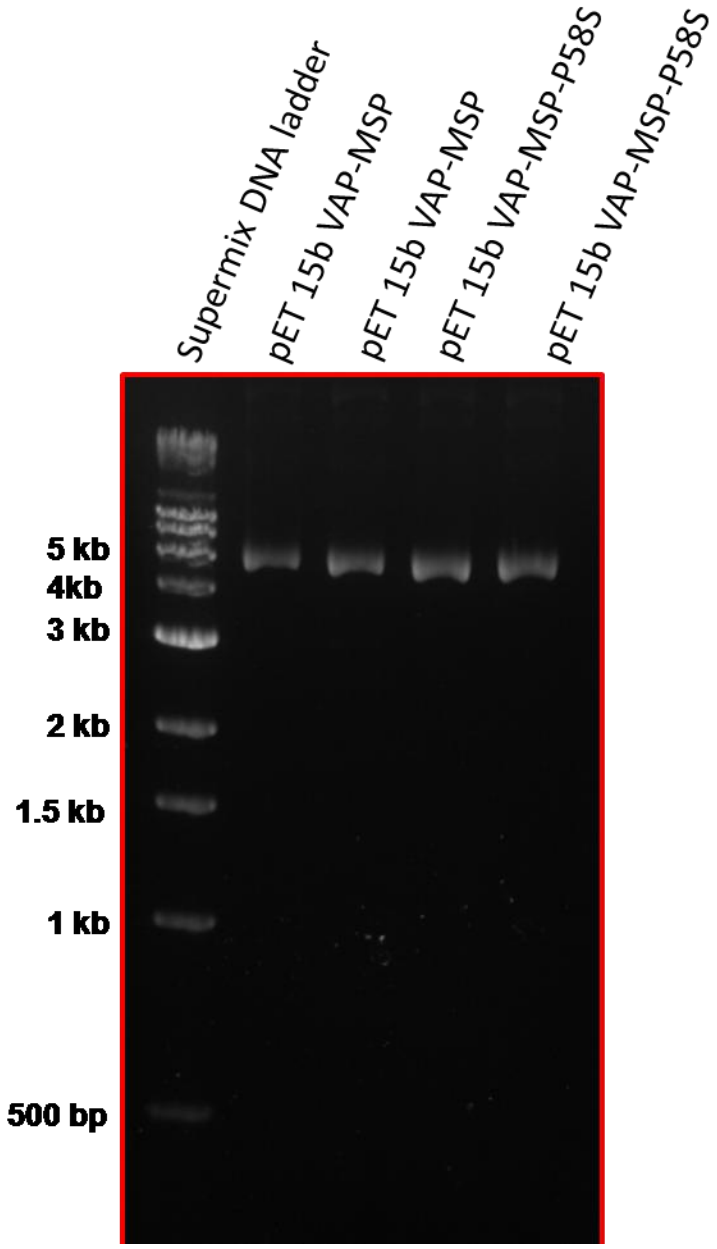

Figure 11: Positive clones for His-tagged proteins

The gel shows two of the many positive clones that were screened for each wild type and mutant. 0.8% Agarose gel, EtBr stained.

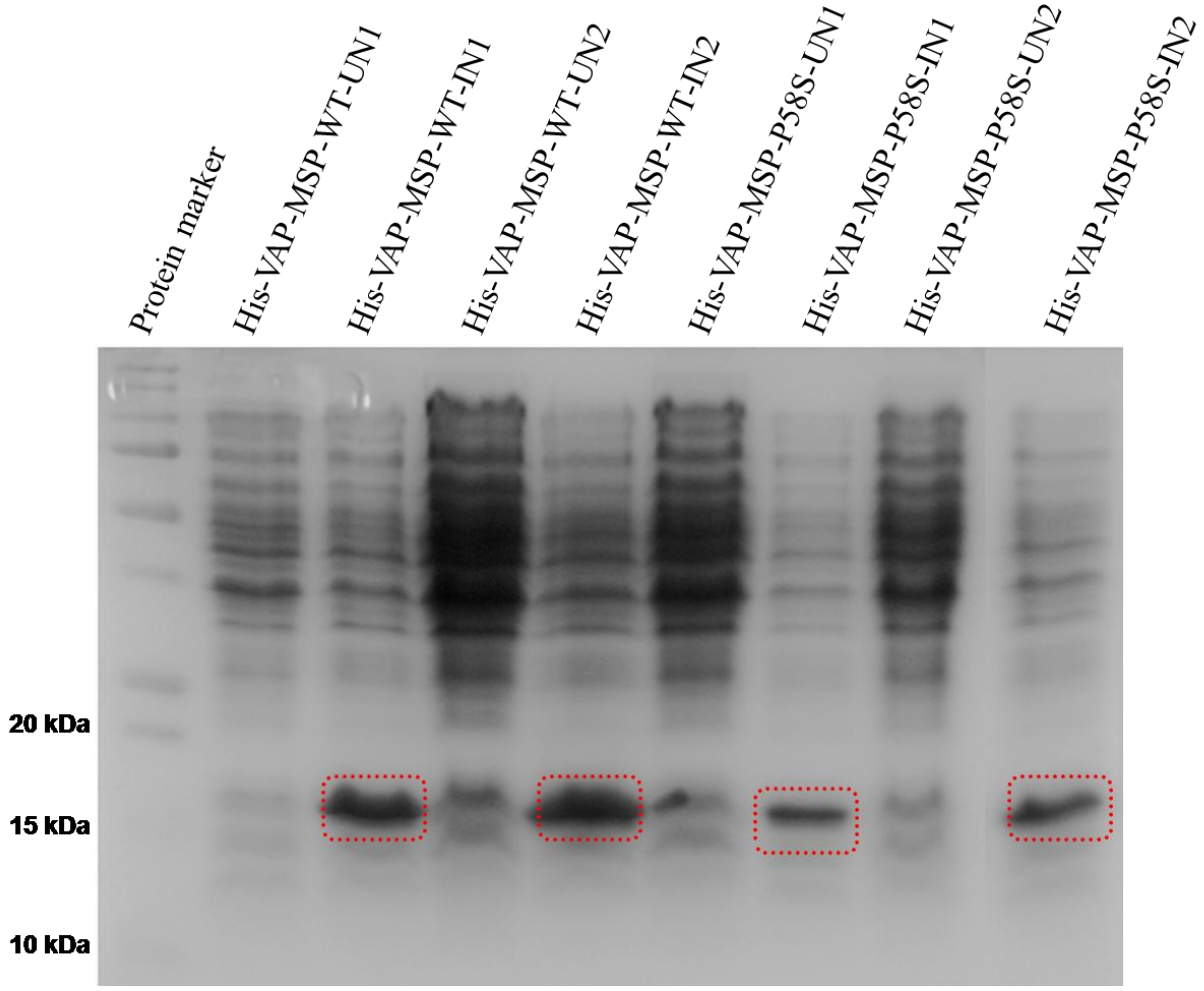

Figure 12: Overexpression of HIS-VAP-MSP wild type and mutant in E. coli

Two sets of each wild type and mutant colonies screened for expression. The dotted squares indicate VAP bands.15% SDS PAGE, CBB stained.

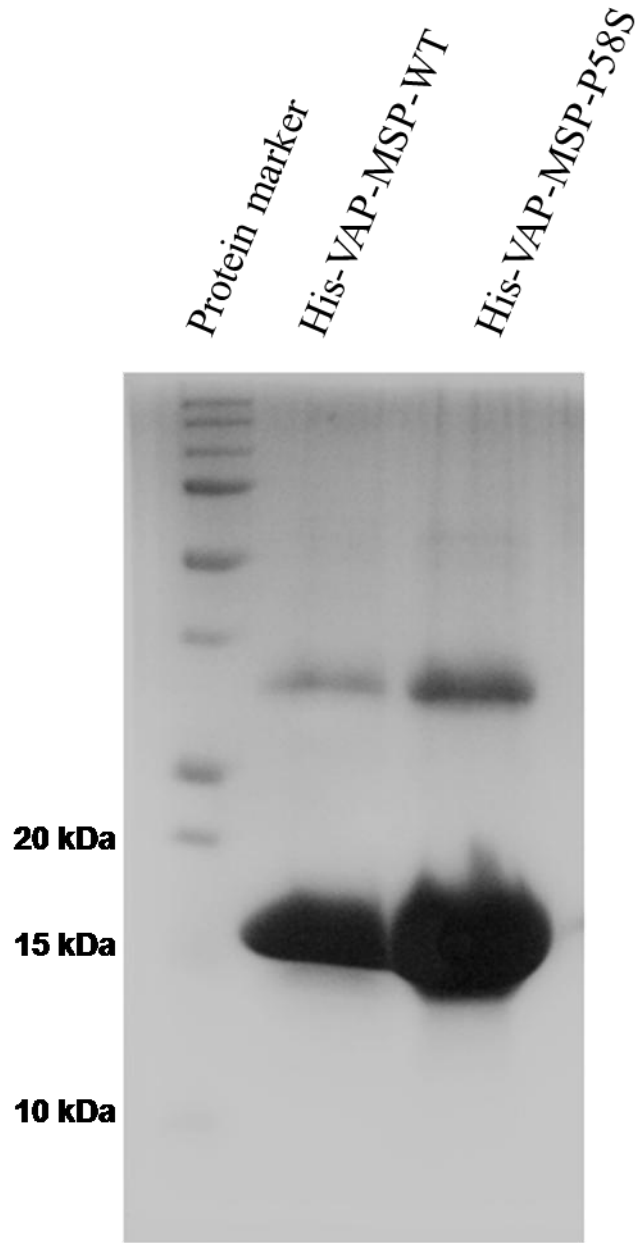

Figure 13: Purified HIS-VAP-MSP wild type and mutant

The overexpressed proteins were purified using Ni-NTA beads under denaturing condition (8M urea) in presence of 50mM Imidazole and eluted at 350mM Imidazole concentration.15% SDS PAGE, CBB stained.

Protein pull-down from *Drosophila*

Drosophila lines containing Myc tagged VAP (wt) and VAP (P58S) were crossed with *elav*-Gal4 driver on II chromosome. The F1 flies were tested for expression by western blotting **(Figure 14)**. Stable lines overexpressing VAPs made by Senthilkumar D were also show to be expressing proteins as tested by western blotting (data not shown). These stable lines are now being used for co-immunoprecipitaion to find out the interacting proteins of VAP *in-vivo*. Previous studies that have identified physical interactors of VAP have been done *in-vitro* by purifying the protein separately and pulling down interactors from a lysate (Mitne-Neto et al., 2007). We expect that pulling down proteins directly from the tissue of interest and using a full length protein will give the advantage of identifying more and reliable interactors.

Senthilkumar D, a graduate student in the lab has completed a reverse genetic screen for interactors of VAP. He has screened 20% of the *Drosophila* genome, identifying 132 enhancers/suppressors. The physical interactor screen will be complementary to Senthilkumar's genetic screen. Senthilkumar has also developed an antibody against the CCD domain of VAP. This antibody along with the α-Myc and α-HA mouse antibodies (Sigma) will be useful tools *in-vitro* and *in-vivo* for our planned experiments.

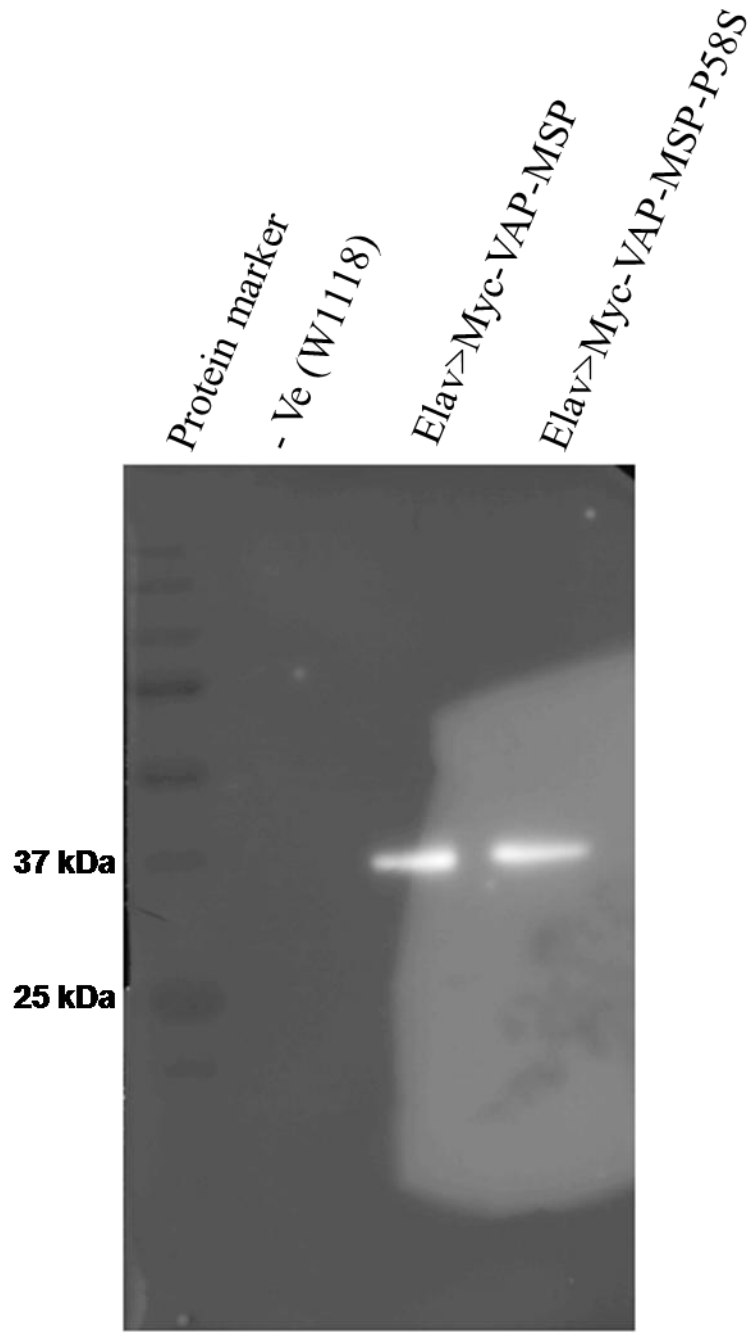

Figure 14: Western blotting confirming expression of Myc tagged VAP-MSP wt and P58S in Drosophila nervous system

Drosophila head lysates were loaded on a *12% SDS PAGE gel and proteins blotted to a PVDF membrane*. *Primary antibody monoclonal used was α-Myc mouse IgG 1:1000 and secondary α-mouse HRP 1:10,000*

Summary

Our primary goal in this study was to purify VAP-MSP domain proteins, both wild type and mutant. The proteins were expressed in *E. coli* using the T7 expression system. The purified protein would be used for biophysical characterization and used as bait to identify interacting proteins. Our initial attempts with GST fusion versions of VAP-MSP did not succeed due to aggregation of the proteins and also due to unusual stickiness of the cleaved MSP-domain proteins to the GST sepharose beads. We, next, re-cloned and expressed the proteins as N-terminal His-Tagged proteins. The proteins were successfully expressed and purified without the above complications, though we are still dealing with protein loss by aggregation during refolding. With purified proteins in hand we are poised to do biophysical experiments.

Work in Progress

We plan to wrap up the Biophysical characterization and interaction experiments in the month before the end of the semester. Experiments in progress include:

- Differential pull down of proteins for VAP-MSP (wt) and VAP-MSP (P58S) from *Drosophila* lysates.
- Characterization of the purified, His-tagged proteins. This would include equilibrium denaturation studies and testing aggregation states of the proteins using chromatography.
- Confirm folded, biological state of the protein by studying interaction of these proteins using FFAT containing peptides, possibly by titration calorimetry.
- Affinity purification of expressed, Myc-tagged protein from *Drosophila* lysates. Mass spectrometric analysis of the interactors.

References

Bottino, D., Mogilner, A., Roberts, T., Stewart, M., and Oster, G. (2002). How nematode sperm crawl. Journal of cell science *115*, 367-384.

Bruijn, L.I., Houseweart, M.K., Kato, S., Anderson, K.L., Anderson, S.D., Ohama, E., Reaume, A.G., Scott, R.W., and Cleveland, D.W. (1998). Aggregation and motor neuron toxicity of an ALS-linked SOD1 mutant independent from wild-type SOD1. Science *281*, 1851-1854.

Bruijn, L.I., Miller, T.M., and Cleveland, D.W. (2004). Unraveling the mechanisms involved in motor neuron degeneration in ALS. Annual review of neuroscience *27*, 723-749.

Chai, A., Withers, J., Koh, Y.H., Parry, K., Bao, H., Zhang, B., Budnik, V., and Pennetta, G. (2008). hVAPB, the causative gene of a heterogeneous group of motor neuron diseases in humans, is functionally interchangeable with its Drosophila homologue DVAP-33A at the neuromuscular junction. Human molecular genetics *17*, 266-280.

Chen, H.J., Anagnostou, G., Chai, A., Withers, J., Morris, A., Adhikaree, J., Pennetta, G., and de Belleroche, J.S. (2010). Characterization of the properties of a novel mutation in VAPB in familial amyotrophic lateral sclerosis. The Journal of biological chemistry *285*, 40266-40281.

Chintapalli, V.R., Wang, J., and Dow, J.A. (2007). Using FlyAtlas to identify better Drosophila melanogaster models of human disease. Nature genetics *39*, 715-720.

Ferraiuolo, L., Kirby, J., Grierson, A.J., Sendtner, M., and Shaw, P.J. (2011). Molecular pathways of motor neuron injury in amyotrophic lateral sclerosis. Nature reviews Neurology *7*, 616-630.

Kaiser, S.E., Brickner, J.H., Reilein, A.R., Fenn, T.D., Walter, P., and Brunger, A.T. (2005). Structural basis of FFAT motif-mediated ER targeting. Structure *13*, 1035-1045.

Lacomblez, L., Bensimon, G., Leigh, P.N., Guillet, P., and Meininger, V. (1996). Dose-ranging study of riluzole in amyotrophic lateral sclerosis. Amyotrophic Lateral Sclerosis/Riluzole Study Group II. Lancet *347*, 1425-1431.

Lev, S., Ben Halevy, D., Peretti, D., and Dahan, N. (2008). The VAP protein family: from cellular functions to motor neuron disease. Trends in cell biology *18*, 282-290.

Lin, D.M., and Goodman, C.S. (1994). Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance. Neuron *13*, 507-523.

Loewen, C.J., and Levine, T.P. (2005). A highly conserved binding site in vesicle-associated membrane protein-associated protein (VAP) for the FFAT motif of lipid-binding proteins. The Journal of biological chemistry *280*, 14097-14104.

Loewen, C.J., Roy, A., and Levine, T.P. (2003). A conserved ER targeting motif in three families of lipid binding proteins and in Opi1p binds VAP. The EMBO journal *22*, 2025-2035.

Mitne-Neto, M., Ramos, C.R., Pimenta, D.C., Luz, J.S., Nishimura, A.L., Gonzales, F.A., Oliveira, C.C., and Zatz, M. (2007). A mutation in human VAP-B--MSP domain, present in ALS patients, affects the interaction with other cellular proteins. Protein expression and purification *55*, 139-146.

Naganska, E., and Matyja, E. (2011). Amyotrophic lateral sclerosis - looking for pathogenesis and effective therapy. Folia neuropathologica / Association of Polish Neuropathologists and Medical Research Centre, Polish Academy of Sciences *49*, 1-13.

Nishimura, A.L., Mitne-Neto, M., Silva, H.C., Richieri-Costa, A., Middleton, S., Cascio, D., Kok, F., Oliveira, J.R., Gillingwater, T., Webb, J.*, et al.* (2004). A mutation in the vesicle-trafficking protein VAPB causes late-onset spinal muscular atrophy and amyotrophic lateral sclerosis. American journal of human genetics *75*, 822-831.

Nishimura, Y., Hayashi, M., Inada, H., and Tanaka, T. (1999). Molecular cloning and characterization of mammalian homologues of vesicle-associated membrane protein-associated (VAMP-associated) proteins. Biochemical and biophysical research communications *254*, 21-26.

Park, D.W., Kim, S.S., Nam, M.K., Kim, G.Y., Kim, J., and Rhim, H. (2011). Improved recovery of active GST-fusion proteins from insoluble aggregates: solubilization and purification conditions using PKM2 and HtrA2 as model proteins. BMB reports *44*, 279-284.

Ratnaparkhi, A., Lawless, G.M., Schweizer, F.E., Golshani, P., and Jackson, G.R. (2008). A Drosophila model of ALS: human ALS-associated mutation in VAP33A suggests a dominant negative mechanism. PloS one *3*, e2334.

Reaume, A.G., Elliott, J.L., Hoffman, E.K., Kowall, N.W., Ferrante, R.J., Siwek, D.F., Wilcox, H.M., Flood, D.G., Beal, M.F., Brown, R.H., Jr.*, et al.* (1996). Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. Nature genetics *13*, 43-47.

Shi, J., Lua, S., Tong, J.S., and Song, J. (2010). Elimination of the native structure and solubility of the hVAPB MSP domain by the Pro56Ser mutation that causes amyotrophic lateral sclerosis. Biochemistry *49*, 3887-3897.

Studier, F.W. (2005). Protein production by auto-induction in high density shaking cultures. Protein expression and purification *41*, 207-234.

Tao, H., Liu, W., Simmons, B.N., Harris, H.K., Cox, T.C., and Massiah, M.A. (2010). Purifying natively folded proteins from inclusion bodies using sarkosyl, Triton X-100, and CHAPS. BioTechniques *48*, 61-64.

Tsuda, H., Han, S.M., Yang, Y., Tong, C., Lin, Y.Q., Mohan, K., Haueter, C., Zoghbi, A., Harati, Y., Kwan, J.*, et al.* (2008). The amyotrophic lateral sclerosis 8 protein VAPB is cleaved, secreted, and acts as a ligand for Eph receptors. Cell *133*, 963-977.

Appendix

Auto induction

Non-inducing medium: ZY broth, 50X M (Buffer), 250X Mg-Metals, 100x 505

Inducing medium: ZY broth, 50X M (Buffer), 250X Mg-Metals, 100x 5052

Trace metal mix:

Component	1x conc
H_2O	-
0.1 M $FeCL_3 \cdot 6H_2O$ (dissolved in ~0.1 M HCl = 100-fold dil of concHCl)	50 µM Fe
1M $CaCl_2$	20 µM Ca
1M $MnCl_2 \cdot 4H_2O$	10 µM
1 M $ZnSO_4 \cdot 7H_2O$	10 µM Zn
0.2 M $CoCl_2 \cdot 6H_2O$	2 µM Co
0.1 M $CuCl_2 \cdot 2H_2O$	2 µM Cu
0.2 M $NiCl_2 \cdot 6H_2O$	2 µM Ni
0.1 M $Na_2MoO_4 \cdot 2H_2O$	2 µM Mo
0.1 M $Na_2SeO_3 \cdot 5H_2O$	2 µM Se
0.1 M H_3BO_3	2 µM H_3BO_3

5052: 0.5 % glycerol, 0.05% glucose, 0.2% α-lactose

505: 0.5 % glycerol, 0.05% glucose

50 X M (Phosphate buffer)

Component	100 ml	1 liter	mol/liter
dd H_2O	80 ml	800 ml	-
Na_2SO_4	3.55 g	35.5 g	5mM
KH_2PO_4	17.0 g	170 g	25mM
Na_2HPO_4	17.75 g	177.5 g	25mM
NH_4Cl	13.4g	134g	50mM

250x Mg-Metals:

Component	10mL	100mL
1M Tri-sodium citrate	2.5mL	25mL
1000X metals	2.5mL	25mL
1M $MgSO_4$	5mL	50mL

ZY Broth: 1% tryptone (w/v), 0.5% yeast extract

Protein Expression and purification

TBS**:** Tris-Cl 50mM; NaCl 150mM; pH 8.

PBS (for thrombin cleavage):

NaCl 140mM; KCl 2.7mM; Na_2HPO_4 10mM; kH_2PO_4 1.8mM; pH 7.3

Lysis buffer (TBS)

Tris buffered Saline (TBS)	1X
Triton X-100	1-2%
Dithiothriotol (DTT)	1mM
(PMSF)	1mM

Lysis buffer 'A': Na_2HPO_4 100mM; Tris-Cl 10mM; pH 8.

For denaturing condition 8 M urea added to lysis buffer 'A'

Thrombin cleavage

1 unit gives more than 90% digestion for 100µg protein in 16-18hrs/22°C.

Thrombin storage buffer:

Sodium citrate	50 mM
NaCl	200 mM
PEG-8000	0.1%
Glycerol	50%

Thrombin cleavage buffer:

$CaCl_2$	5 mM
NaCl	150 mM
Tris-HCl (pH 7.5)	50 mM