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The role of Ser497 in Phosphopantetheinylation-of Tcp11, an L-Hydroxyphenylglycine activating module

Research Project Report

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Abstract

Nonribosomal peptides synthetases (NRPS) are multimodular biocatalysts that build diverse pool of biologically active natural compounds. Nonribosomal peptides (NRPs) are commonly used as antibiotics, antitumor, antifungal or as immunosuppressant. Within multimodular structure of NRPSs, each module comprises of several domains, such as adenylation (A), thiolation (PCP), condensation (C), and thioesterase (TE) domain. One of the important domains is thiolation (PCP) domain, as a strictly conserved serine residue (Ser497) acts in the active site to allow Ppant-arm formation. During this project, another two serine residues (Ser499 and Ser500), located downstream of the conserved Ser497 in PCP-domain of L-Hydroxyphenylglycine activating module (Tcp11) were included in the study. In order to investigate the role of Ser497, the neighboring Ser499 and Ser500 in Ppant-arm formation of Tcp11, a mutagenesis strategy was employed. Therefore single mutations were performed of the serine residues into alanine. Additionally, a triple serine knockout was prepared where all three serine residues were exchanged to alanine (S497A/S499A/S500A). Subsequently, the wild type and the mutants were expressed in *E.coli* and their adenylation and thiolation activity were tested.

Contents

1. Introduction

Nonribosomal Peptide Synthetases (NRPS) (figure 1), are very large enzymes that synthesized peptides (NRPs) or peptides-like products by using thiotemplate mechanism instead of mRNA (Stein, et al. 1996). NRPs are commonly produced in several bacteria and fungi as secondary metabolites by consecutive condensation of amino acids, which is achieved by these multimodular enzymes (Finking en Marahiel 2004). In NRPS system, plenty of amino acids can be used to build a new compound, include 20 proteinogenic amino acid and large number of nonproteinogenic amino acids. To date more than 422 monomers are known to assemble by NRPS enzymes (Essen 2009). Cyclosporine is the first NRP found in fungal. This immunosuppressant drug is synthesized by single 1.6 MDa NRPS (K. Turgay 1992). NRPS system can assemble peptides containing unique structural features such as D-amino acids, N-terminally attached fatty acid chains, N- and C-methylated amino acids, N-formylated residues, heterocyclic elements, glycosylated amino acids, and phosphorylated residues (Essen 2009). An interesting example of NRPS is L-δ -(α –Aminoadipic acid)-L-Cysteine-D-Valine Synthetase (ACVS) which is a multifunctional enzyme known to catalyze condensation of L-δ -(α –Aminoadipic acid), L-Cysteine, and D-Valine into ACV-tripeptide. This tripeptide is known as a precursor of penicillin and cephalosporin antibiotics (Michael F. Byford 1997).

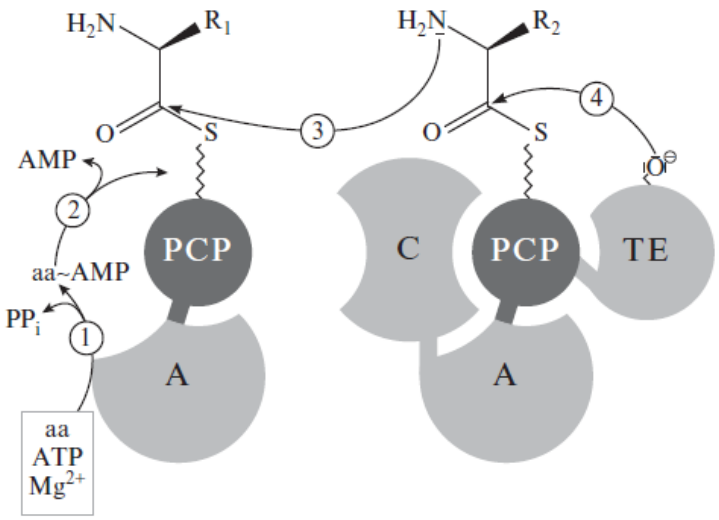

Figure 1. The mechanism of NRPS (Essen 2009). (1) Both ATP and Mg^{2+} are needed to activating the first module, before the amino acylated substrate (AA) will bound to the AMP and release PP_i. Moreover, the amino acylated substrate will tethered to holo form-PCP, which already has phosphopantetheine arm attached on it (2). Furthermore, (3) the C-domain link the other AA to the previous AA by the peptide bound. Lastly, (4) Thioesterase domain releases NRPS product.

Basically, NRPS consists of 3 or more modules, and each module of NRPSs consists necessarily of at least 2 ubiquitous domains (figure 2), adenylation (A) domain, peptidyl carrier protein (PCP) domain, condensation (C) domain, thioesterase (TE) domain and several optional domain, such as epimerization (E) domain, cyclization (Cy) domain, and oxidation (Ox) domain.

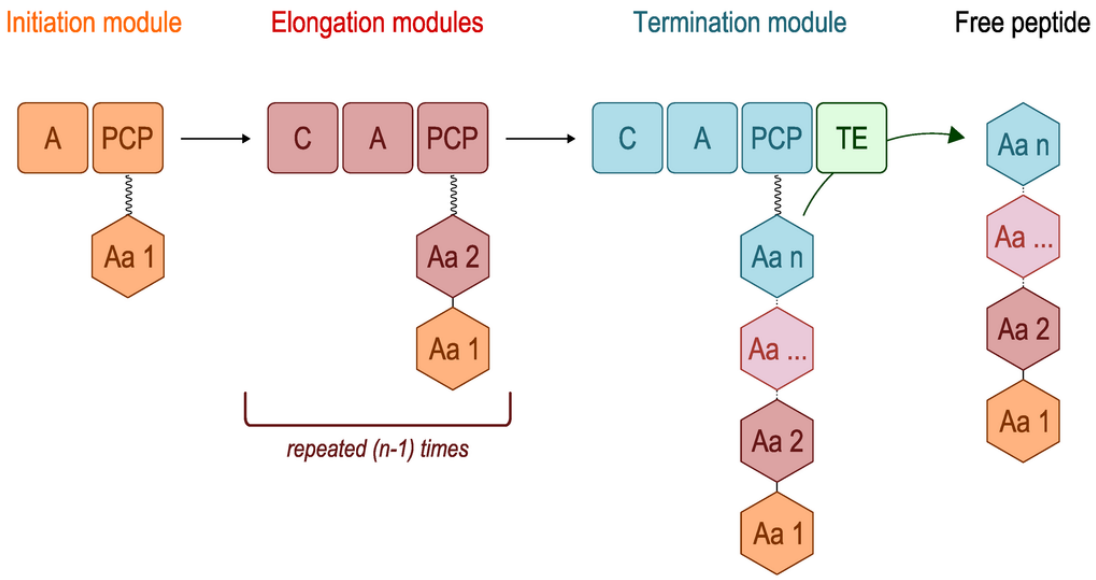

Figure 2. Different modules in NRPS and pathway to release the free-peptide (Desriac, et al. 2013).

A-domain control the entry of the substrates into nonribosomal peptide synthesis by selection of amino acid substrates, and their simultaneous activation as aminoacyl adenylates by the expense of ATP (Rahu, et al. 2013). In the following step, the aminoacyl adenylated substrates are transferred to the (holo) PCP-domain, which 4'-Phosphopantetheine arm already attached to serine residue of the domain. Afterwards, the formation of peptide bond is carried out by the C-domain. According to this model, the organization of the domain within a module is C-A-PCP, and these modules are termed as elongation modules. Furthermore, the growing peptide chain is transferred sequentially to the downstream modules and finally to a termination module where the linear intermediate undergoes macrocyclization and is released by the TE-domain (Rahu, et al. 2013). In addition to these three core domains, modules can contain extra or alternative domains which introduce a modification of the amino acid being incorporated, such as a change of the Cα-stereochemistry (epimerization domain), an N-methylation (N-methylation domain) of the α-amino group, or the heterocyclization of residues.

The PCP-domain comprises of 80-100 amino acids and is located downstream of the A-domain. Within each PCP-domain, a highly conserved serine residue is posttranslationally modified with the *Sfp* (phosphopantetheinyl transferase) by the expense of coenzyme A (CoA) (Essen 2009). The Coenzyme A will break down and release phosphopantetheine and 3', 5'-Phosphoadenosine Phosphate (3', 5'-PAP). Afterwards, the 4'-phosphopanthetheine prosthetic group will covalently attach to the catalytic site (serine residue) in the PCP-domain (Figure 3).

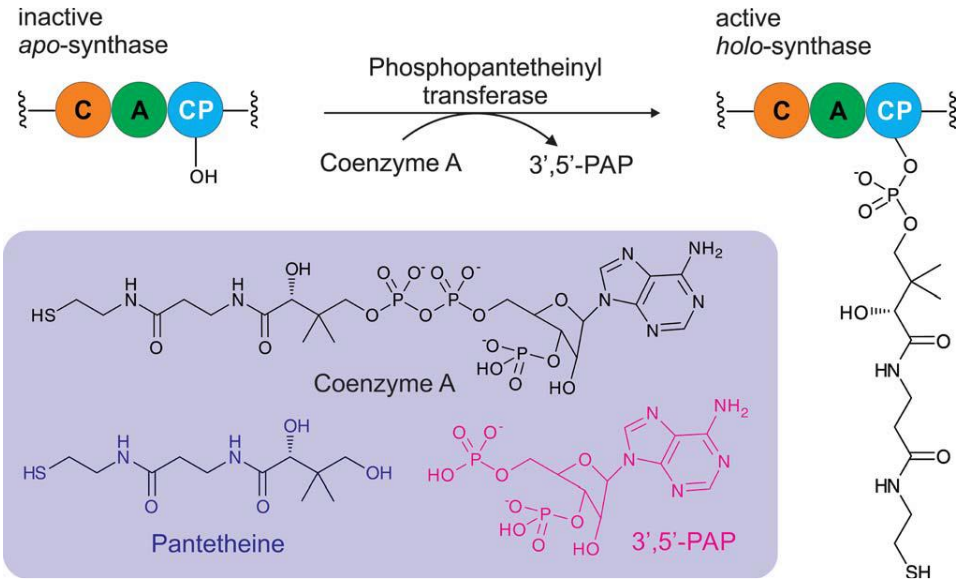

Figure 3. Phosphopantetheinyl transferase convert from inactive form (Apo) to active form (Holo) (Beld, et al. 2014).

The aim of this project is to study the Tcp11 activating module, which activates L-Hydroxyphenylglycine (L-Hpg). The gene that encodes Tcp11 is derived from *Actinoplanes teichomyceticus*, which produces tecoplanin, a glycopeptide antibiotic. This module consists of 3 domains: A-domain, T-domain, and E-domain (figure 4). To overexpress the three-domain protein; the helper protein gene (tcp13) is needed. The helper protein is known to activate A-domain and as well as to enhance the expression of corresponding module.

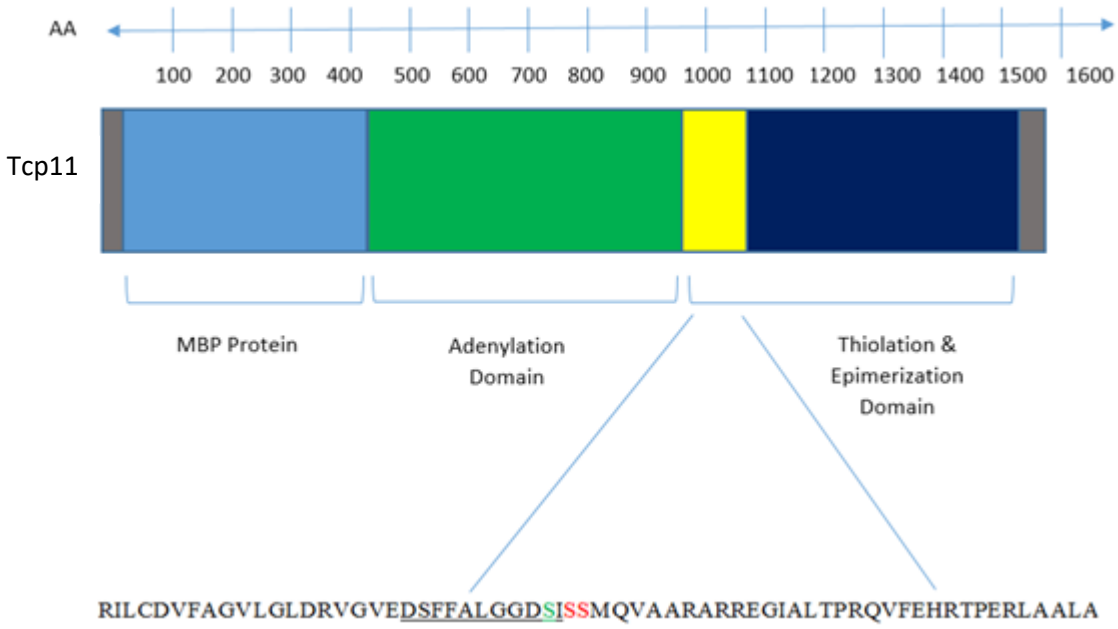

Figure 4. Tcp11 Construct. This construct consists of 6 histidine-tag (grey color in both N and C terminal); MBP protein; A, T- and E-domain. The underlined shows the conserved motif from PCP-domain. The first serine (green) is the catalytic site for 4'-phosphopantetheinylation. Two serine residues (red) are located downstream of Ser497 were included in the mutagenesis strategy.

domain[a]	core(s)[b]	consensus sequence
adenylation	A1	L(TS)YxEL
	A2 (core 1)	LKAGxAYL(VL)P(LI)D
	A3 (core 2)	LAYxxYTSG(ST)TGxPKG
	A4	FDxS
	A5	NxYGPTE
	A6 (core 3)	GELxIxGxG(VL)ARGYL
	A7 (core 4)	Y(RK)TGDL
	A8 (core 5)	GRxDxQVKIRGxRIELGEIE
	A9	LPxYM(IV)P
	A10	NGK(VL)DR
thiolation	T (core 6)	DxFFxxLGG(HD)S(LI)
condensation	C1	SxAQxR(LM)(WY)xL
	C2	RHExLRTxF
	C3 (His)	MHHxISDG(WV)S
	C4	YxD(FY)AVW
	C5	(IV)GxFVNT(QL)(CA)xR
	C6	(HN)QD(YV)PFE
	C7	RDxSRNPL
thioesterase	TE	G(HY)SxG
epimerization	E1	PIQxWF
	E2 (His)	HHxISDG(WV)S
	E3 (race A)	DxLLxAxG
	E4 (race B)	EGHGRE
	E5 (race C)	RTVGWFTxxYP(YV)PFE
	E6	PxxGxGYG
	E7 (race D)	FNYLG(QR)
N-methylation	M1 (SAM)	VL(DE)GxGxG
	M2	NELSxYRYxAV
	M3	VExSxARQxGxLD

Table 1. Figures highly conserved core motif of peptide synthetases from different domains (Marahiel, Stachelhaus and Mootz 1997).

The Tcp11 module comprising of A-, T- and E-domains was constructed as fused protein with Maltose Binding Protein (MBP) at N-terminus. The overexpressed protein was fused with MBP to increase the solubility of recombinant expressed in *E.coli*. Meanwhile, the mechanism of MBP increase solubility still not really well understood (Raran-Kurussi en Waugh 2012). Upstream of the MBP gene there is histidine-tag added, which allows purification by metal-affinity chromatography (His-tag purification).

During this project, the conserved and non-conserved serine residues located in the active site of PCP-domain were mutated into alanine. Ppant-arm attachment occurs via the hydroxyl group of serine. Thus the methyl group from alanine will make impossible Ppant-arm anchoring. Afterwards, Pyrophosphate assay was performed as an initiating test to observe the catalytic site of A-domain. The mutation in the PCP-domain should not prevent aminoacyl adenylate process in the A-domain. This assay measures amount of pyrophosphate released after activation of the A-domain. This reaction consists of 2 stages: Firstly, the AMP is formed by hydrolysis of ATP and inorganic phosphate (PPi) as a byproduct. The AMP is bound to the activated substrate as amino-acylated intermediate Hpg-O-AMP. Secondly, the AMP-substrate complex will tether to PCP-domain. The amount of released PPi created a complex reaction with MESG from the kit assay, and measured in 360nm (figure 5).

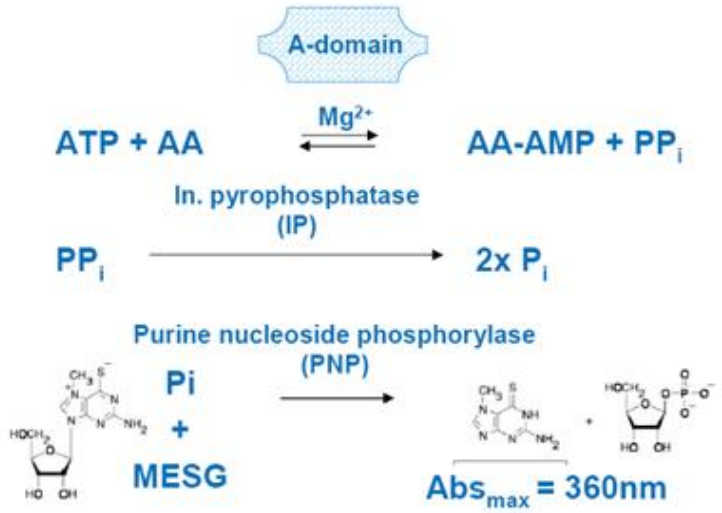

Figure 5. Schematic view of Pyrophosphate assay in A-domain.

Trypsin is a serine protease, commonly found in the digestive system of human and many vertebrates (Mesuere 2015). This protease used for protein digestion, and LC-MS analysis based proteomics since it has a well-defined specificity; it hydrolyzes only the peptide bonds in which the carbonyl group is followed either by an arginine (Arg, R) or lysine (Lys, K) residue (figure 6), with the exception when Lys and Arg are N-linked to Aspartic acid (Asp).

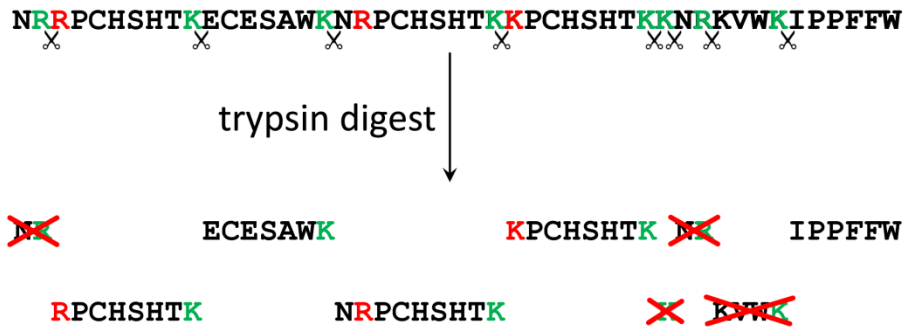

Figure 6. Trypsin digestion cutting sequences (Mesuere 2015)

2. Materials and methods

- **Preparation of *E.coli* DH5α and BL21 Competent Cells**

See the protocol

- **Mutagenesis studies on Tcp11 (L-Hpg activating module)**

DNA Mutation	Primer Info & PCR setting
Hpg22 ATE S497A:	FW1 S497A: 5' – phosphorylated CTATTAGCAGCATGCAGGTTGCAG – 3' RV1 S497A: 5' – CATCACCACCCAGTGCAAAAAAGC – 3' PCR Setting: Initial denaturation 98°C 30s Denaturation 98°C 15s Annealing 56-59°C 30s 30 cycles Elongation 72°C 4m 45s Final Extension 72°C 10m
Hpg22 ATE S499A:	FW2 S499A: 5' – phosphorylated CTAGCATGCAGGTTGCAGCACGTG – 3' RV2 S499A: 5' – CAATGCTATCACCACCCAGTGCA – 3' PCR Setting: Initial denaturation 98°C 30s Denaturation 98°C 15s Annealing 63-66°C 30s 30 cycles Elongation 72°C 4m 45s Final Extension 72°C 10m
Hpg22 ATE S500A	FW3 S500A: 5' – phosphorylated CTATGCAGGTTGCAGCACGTGC – 3' RV3 S500A: 5' – CGCTAATGCTATCACCACCCAGTG – 3' PCR Setting: Initial denaturation 98°C 30s Denaturation 98°C 15s Annealing 63-66°C 30s 30 cycles Elongation 72°C 4m 45s Final Extension 72°C 10m
Hpg22 ATE S497A/S499A/S500A	FW4 S497A/S499A/S500A : 5' – phosphorylated GCTATGCAGGTTGCAGCACGTG – 3' RV4 S497A/S499A/S500A : 5' – CGCAATAGCATCACCACCCAGTGC – 3' PCR Setting: Initial denaturation 98°C 30s Denaturation 98°C 15s Annealing 63-66°C 30s 30 cycles Elongation 72°C 4m 45s Final Extension 72°C 10m

Table 2. Primer DNA Mutation Hpg22 ATE S497A; S499A; S500A; S497A/S499A/S500A

- **Cloning strategy**

In order to remove the template plasmid, the obtained PCR product was incubated with DpnI enzyme, which will digest methylated DNA. Afterwards, all PCR products were ligated using T4 DNA ligase (blunt end ligation with 5'-phosphorylated primer).

- **Transformation to *E.coli* DH5α**

Following ligation reaction, the DNA was successfully transformed into *E.coli* DH5α competent cells. The cells were plated on LB-agar supplemented with kanamycin. The grown colonies were picked for DNA plasmid isolation step.

- **Plasmid DNA isolation**

The overnight culture was used to perform a miniprep plasmid DNA isolation according to the protocol.

- **Co-transformation to *E.coli* BL21 (DE3) with tcp13 helper protein gene**

Both DNA plasmids pET containing Tcp11 gene and pACYC containing tcp13 gene were co-transformed into *E.coli* BL21 (DE3) competent cells for protein expression. This helper protein is a constructed plasmid without the histidine$_6$-tag to diminish the inappropriate number of helper protein trapped during purification then cells were plated on LB-agar supplemented with kanamycin and chloramphenicol. The growing colonies were picked for further overexpression tests

- **Overexpression and co-purification of Tcp11 with tcp13**

E.coli BL21 containing Tcp11 and tcp13 proteins were inoculated in 5 ml LB medium supplemented with kanamycin and chloramphenicol and grown over night at 37°C and 200 rpm. Out of this overnight culture, 1ml medium was used to inoculate into 100 ml of 2xPY medium (supplemented with kanamycin and chloramphenicol). The cell culturing was performed at 37°C and 200 rpm to an OD_{600} of 0.5-0.6. Once the proper OD value is reached, the temperature was adjusted to 18°C and protein was induced by adding 0.5mM IPTG. Subsequently, cell culturing was continued overnight at 200 rpm.

The overnight culture was spun down at 3,000-4,000g at 4°C for 15 minutes. Then the supernatant was discarded and pellet was resuspended in 1ml lysis buffer for every 100 ml culture and put on ice. Afterwards, cells were disrupted using sonicator device. The following parameters are applied to the machine: 40 cycles-6sec ON/ 15 sec OFF with amplitude 6-10 microns. After that, the cell lysate is centrifuged at 15-17,000 g for 15 minute at 4°C. The

supernatant was collected for protein purification and the pellet discarded. 10µl of cell free lysate (CFL) is kept for SDS PAGE analysis.

Next, the Ni-NTA column can be prepared; approximately 300 µl resin was required. The resin was equilibrated in a 15ml tube with 10ml equilibration buffer. After that, the tube was incubated at 4°C and gently shaking for 5 minutes before centrifuged at 800g for 5 minutes. The supernatant was then discarded and replaced by 10ml fresh equilibration buffer and repeat this step again.

Then, cell free lysate was loaded onto the column. The lysate is added and the total volume of the mixture is adjusted to 10ml with equilibrium buffer and kept on a gentle shaker at 4°C for 1 hour, the column was then positioned vertically to allow the resin beds settled down.

After it settled, the cap and lid can be removed and the cell free lysate passes through. After the last wash step, put the cap on the bottom and add the elution buffer with 800 µl of lowest concentration imidazole (100 mM), then keep it at 4°C for 15 minutes. Protein sample was eluted in 1.5ml Eppendorf tubes by applying different concentration of imidazole in the elution buffer (175 and 250 mM) can be added to the column with same procedure. Subsequently, the eluted protein fractions were concentrated by using Amicon Ultra 100 (100 kDa membrane) filters. Lastly, 10% glycerol can be added for long term storage of the protein at -20°C.

- **Pyrophosphate Assay**

The following mastermix reaction for pyrophosphate assay:

Pyrophosphate Assay	Volume (µl)
1M $MgCl_2$	1
100 mM ATP	5
1 mM MESG	40
IP 30U/ml	1
PNP (100 U/ml)	1
1 M DTT	7.5
50 mM HEPES pH 8.0	14.5

Table 3. Mastermix composition for pyrophosphate assay.

Once all of the component are mixed, 70µl of this mastermix was added to the dedicated well on 96 well plate (UV-transparent). Subsequently, 20 µl of the enzyme was added to prepared wells and the plate is incubated at room temperature for 10-15 minutes. After that, 10µl of 10µM substrate was added to the prepared wells and measurement at 30°C was started immediately. The setting of the measurement is 4 hours with time reads at 360nm points every 5 minutes. This assay requires a negative (Phe) and positive control (Hpg) for accurate measurements.

- ***Sfp* assay (Phosphopantetheinylation assay)**

***Sfp* Assay**	**Volume (µl)**
Enzyme (~50 µM)	20
CoA (0.5 mM) stock 10 mM	2.5
MgCl2 (0.5 mM) stock 10 mM	2.5
Sfp (40 µM)	1
HEPES pH7(50 µM) NaCl (300 µM)	23
TCEP (0.5 µM)	1

Table 4. Mixture composition for *Sfp* assay.

***Sfp* Assay and A-domain activation (L-Hpg)**	**Volume (µl)**
Enzyme (~50 µM)	20
CoA (0.5 mM) stock 10 mM	2.5
MgCl2 (0.5 mM) stock 10 mM	2.5
Sfp (40 µM)	1
HEPES pH7(50 mM) NaCl (300 mM)	8
TCEP (0.5 mM)	1
L-Hpg (10 mM)	10
ATP (10 µM)	5

Table 5. Mixture composition for *Sfp* assay with A-domain activation.

Once everything was mixed, 50 µl of mixed reaction was incubated in the room temperature for 1 hour before analyzed in 10% SDS PAGE gel.

- **CoA-fluorophore labeling reaction**

PCP tag reaction	Volume (µl)
Enzyme (~3.8 µM)	20
CoA 647 (250 µM)	4
MgCl2 (1 M)	1
Sfp (40 µM)	1
HEPES pH7(1 M)	1.25
MQ water	21.5
DTT (50 mM)	1

Table 6. Mixture composition for labeling CoA activation

As soon as all components are mixed, 50 µl of reaction was incubated at the 37˚C temperature for 1 hour before analyzed in a 10% SDS PAGE gel. The luminescence was detected with image analyzer, before stained with the coomasie blue.

- **Trypsin digestion and MALDI TOF-MS analysis**

Met G S S H H H H H S S G L V P R G S H Met K I E E G K L V I W I N G D K G Y N G L A E V G K K
F E K D T G I K V T V E H P D K L E E K F P Q V A A T G D G P D I I F W A H D R F G G Y A Q S G L L
A E I T P D K A F Q D K L Y P F T W D A V R Y N G K L I A Y P I A V E A L S L I Y N K D L L P N P P K
T W E E I P A L D K E L K A K G K S A L Met F N L Q E P Y F T W P L I A A D G G Y A F K Y E N G K
Y D I K D V G V D N A G A K A G L T F L V D L I K N K H Met N A D T D Y S I A E A A F N K G E T
A Met T I N G P W A W S N I D T S K V N Y G V T V L P T F K G Q P S K P F V G V L S A G I N A A S P
N K E L A K E F L E N Y L L T D E G L E A V N K D K P L G A V A L K S Y E E E L V K D P R I A A
T Met E N A Q K G E I Met P N I P Q Met S A F W Y A V R T A V I N A A S G R Q T V D E A L K D A Q T
N S S S N N N N N N N N N L G I E G R L Q G P Met L T V A A I D V T S A A E R D R V A R W G A A
V G A R P D R L A L D L F A R Q V A Q R P D E V A V A D G D R V Met S F G E L A E R A D R L A G H
L S A R G V R R G D R V A V V Met E R S G E L I A T L L A V W R A G A A F V P V D P A Y P A E R V
K F L L T D A E P V A A V C T A A F R A A V L D G G L E A I V V D D P G T W P A V A P C P P V P T
G P D D L A Y V Met Y T S G S T G T P K G V A V S H G D V A A L V G D P G W R T G P G D T V
L Met H A S H A F D I S L F E I W V P L L S G A R V Met I A G P G A V D G A A L A A A Q V A A G V T A
A H L T A G A F R V L A E E S P E S V A G L R E V L T G G D A V P L A A V E R V R R A C P D V R V
R H L Y G P T E T T L C A T W W L L E P G D E T G P V L P I G R P L A G R R V Y V L D A F L R P L P
P G T T G E L Y V A G A G V A Q G Y L G R P A L T A E R F V A D P F A P G G R Met Y R T G D L A Y
W T E Q G T L A F A G R A D D Q V K I R G Y R V E P G E V E A V L G G L P G V A Q A V V C V R G E
H L I G Y V V A E A G R D L D P E R L R A R L A A T L P E F Met V P A A V L V L A D L P L T V N G K
V D R P A L P E P D F A A K S T G R A P A T A A E R I L C D V F A G V L G L D R V G V E D S F F A L
G G D S I S S Met Q V A A R A R R E G I A L T P R Q V F E H R T P E R L A A L A P A A G S A R P D R
S A A D A G L G E I P W T P V Met R A L G D D A V R P G F A Q A R V V V A P A G L D P D A L T G A
L R A V L D T H D V L R A R V E P D R R L I V P E R G A V A A A D L L T R V A V D S D G I D A R A
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H W V T V L D A A E P P L A E H H G Q A H S W S A T L S G A V A G H L V S R Met P G A F H C G I Q
E V L L A G L A A A A V A R W R G D D A G V L V D V E G H G R H A A D G E D L L R T V G W F T S V
H P V R L D V S G V D L A A A A A G D A A A G E L L K S V K D Q V R A A P G D G F G F G L L R H L
N P D T A E R L A A L P A P Q I G F N Y L G R S G V A A E A V P W Q V R G G S L G A G E A G P D L
V L A H P L E A G A D V R D T P D G P L L R L T L D G R D L A P V T V E L L G E A W L E L L T G L
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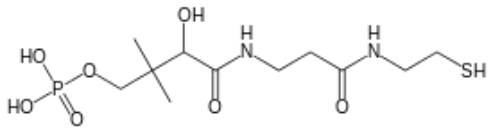
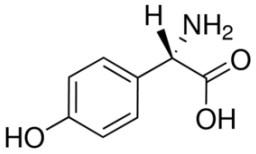


Phosphopantetheine Arm
358.349 g/mol



4-hydroxy-l-phenylglycine
167.16 g/mol

Figure 7. Protein sequence of Tcp11, an Hpg activating module. The red fonts reveal the trypsin specific cleavage site. The residues highlighted in yellow shows the sequence area targeted for mutagenesis. Upon 4'-Phosphopantetheinylation catalyzed by *Sfp*, the mass of the thiolation domain will increase. In addition, the substrate (L-Hpg), will increase mass by 525.5 Da in the holo-form.

3. Results and discussion

The plasmid containing Tcp11 gene was amplified with PCR primers to create 4 different mutants in the PCP-domain (figure 8). In order to improve the yield and specificities of PCR priming reaction, 5% DMSO was applied to PCR mixture (Hardjasa, et al. 2010). Then the amplified amplicons were incubated with DpnI enzyme for digest methylated DNA, and followed by a blunt-end ligation step.

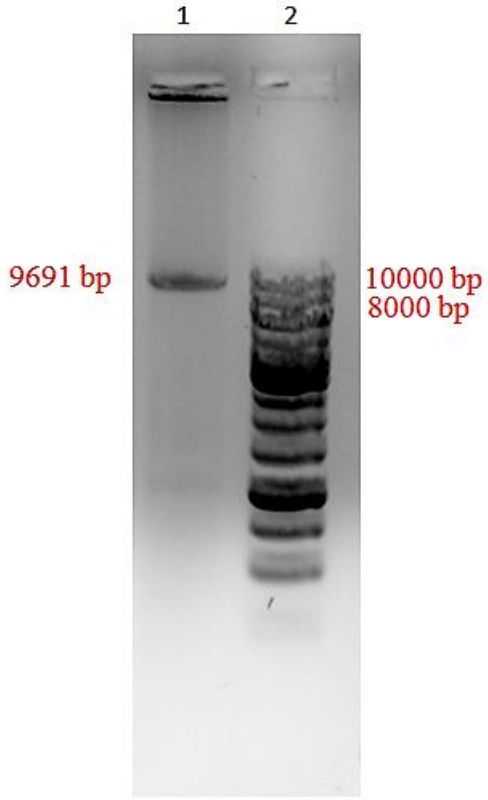


Figure 8. The mutant amplicon (S497A) from Tcp11. The other mutants (S499A; S500A; S497A/S499A/S500A) showed the same figure.

For expression, the plasmid containing Tcp11 gene was co-transformed with another plasmid containing the helper protein gene (tcp13). The Tcp11 mutants and wild type were successfully overexpressed in the *E.coli* BL21 cells (figure 9).

During protein purification step, the cells were resuspended in sonication buffer before lysed. After sonication and centrifugation, the supernatant was purified by affinity chromatography (his-tag purification) to separate the protein from contaminants and cell lysates. The tagged protein can bind the nickel column (Ni-Nta), while contaminants will flow through from the column. The purity of histidine-tagged proteins can be improved by increasing the imidazole concentration during the purification process (figure 9). Hence, the

higher concentration of imidazole means more imidazole that competes with the target protein, before the protein eluted out from the column. In addition, the amylose column can be used as an additional step to purify the protein, since the protein target is fused with MBP, which can be purified by eluting the column with maltose.

As identified in SDS-PAGE gel, the bands from the purified mutant were observed, and the size is same as expected, approximately 162 kDa (figure 9). Afterwards, the mutants were tested with pyrophosphate assay. This assay was performed to investigate adenylation domain activity, by the presence of inorganic phosphate (PP_i) released from amino-acylated substrate. According to the result (figure 10), it showed the adenylation domain can still catalyze substrate selection and activation as aminoacyl-O-AMP for both L-Hpg and D-Hpg with the expense of ATP. The A-domain also known has highly selectivity for the substrate selection. In this assay, Hpg25 was used as another positive control derived from *metagenomics data* source. Consequently, Phenylalanine was used as a negative control.

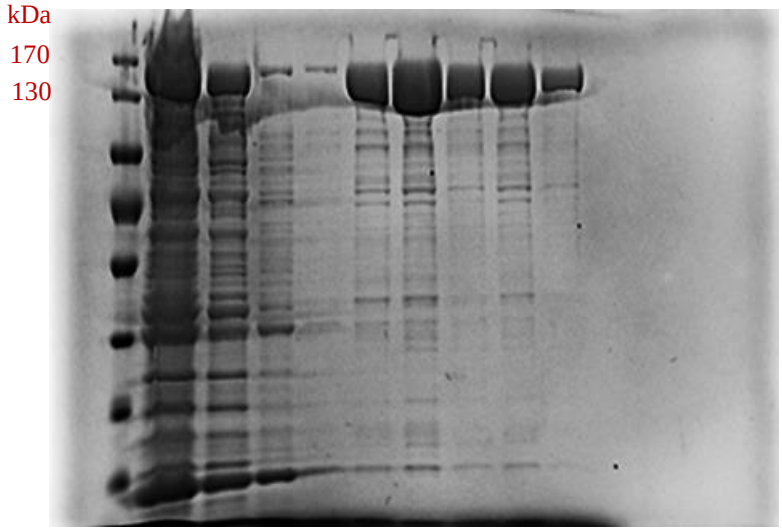


Figure 9. The SDS page gel after overexpression processes. From left: Protein ladder (1); Cell free lysate (2); Flow Through (3); Washing 1(4); Washing 2(5); Elution step with different imidazole concentration (6-8)(100mM; 175mM; 250mM); Concentrated Elution (9,10)(175 mM & 250 mM).

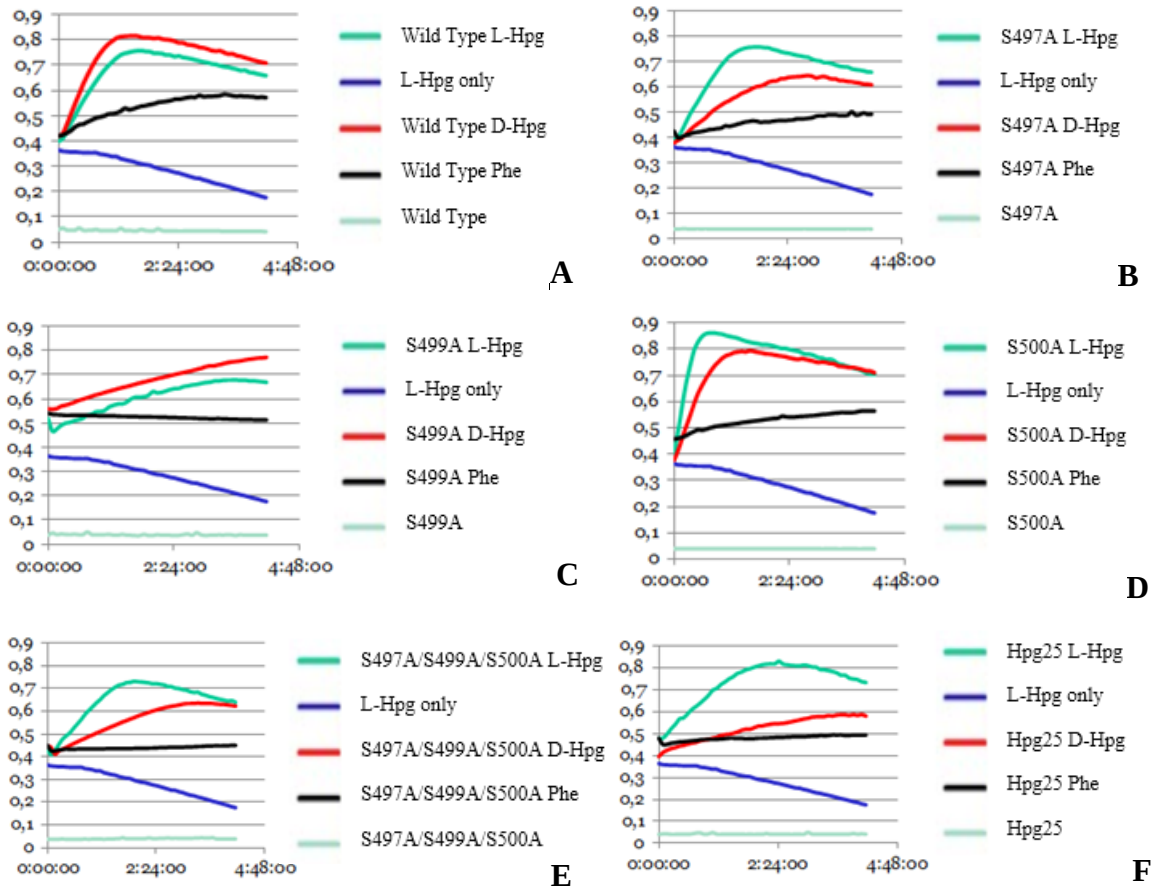


Figure 10. The Pyrophosphate assay result for Tcp11 wild type (A) and Mutants: conserved serine mutant (B), second mutant (C), third mutant (D), triple mutant (E). Hpg25 was used as a positive control (F).

In order to install 4'-phosphopantetheinyl arm in the active serine residue of the PCP-domain, *Sfp* assay was performed. *Sfp* is a highly promiscuous type II PPTase derived from *Bacillus subtilis*, which activates PCPs and FAS apo-ACPs alike dependent with Mg^{2+} ions (Beld, et al. 2014). After treated with *Sfp* reaction (figure 11), the upper band (162 kDa) was cut and treated with trypsin (in gel digestion step). A separate band appeared in the gel at approximately 70 kDa came from the mixture in *Sfp synthase* kit. According to the trypsin digestion result (figure 12), the trypsin does not efficiently cut the protein target sequence, while 80% of the obtained coverage came from MBP part instead of the protein target.

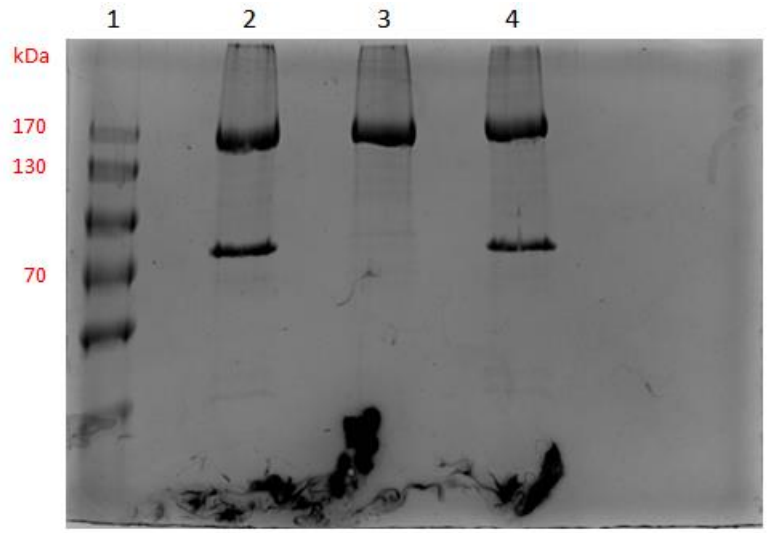


Figure 11. The SDS page of *Sfp* Assay. From left: Protein ladder (1); Enzyme treated with *Sfp* (2); Enzyme only as negative control (3); Enzyme treated with *Sfp* and substrate (L-Hpg)(4).

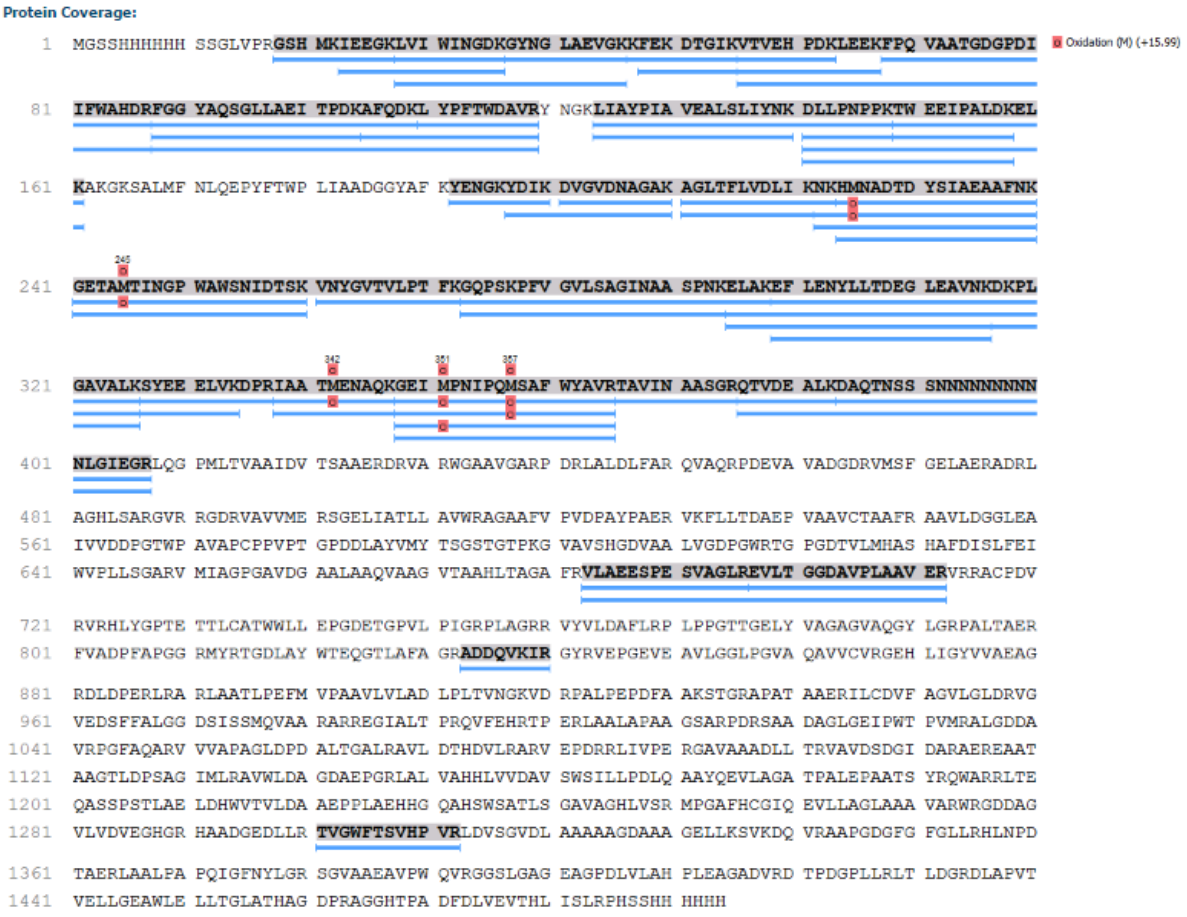


Figure 12. Trypsin digestion result (Wild type Tcp11 fused with MBP construct).

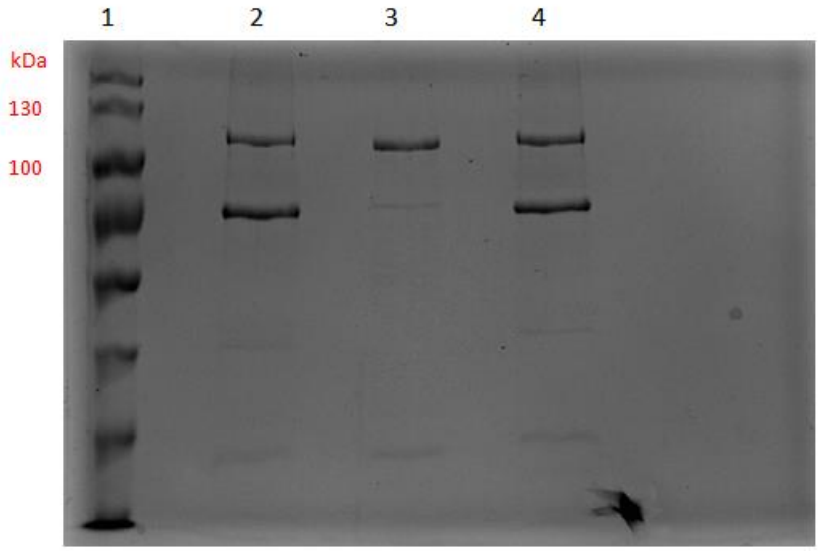


Figure 13. *Sfp* assay of Tcp11 construct (lack of MBP). From left: Protein ladder (1); Enzyme treated with *Sfp* (2); Enzyme treated without *Sfp* (3); Enzyme treated with *Sfp* and substrate (L-Hpg) (4).

Based on this result, few alternative methods could be performed. Firstly, is removing the MBP part from the construct. The band without MBP was 40 kDa smaller. The non-MBP band showed with 120 kDa band (figure 13). As well as, the band was treated with Trypsin (in gel digestion step) before analyzed by MALDI TOF-MS.

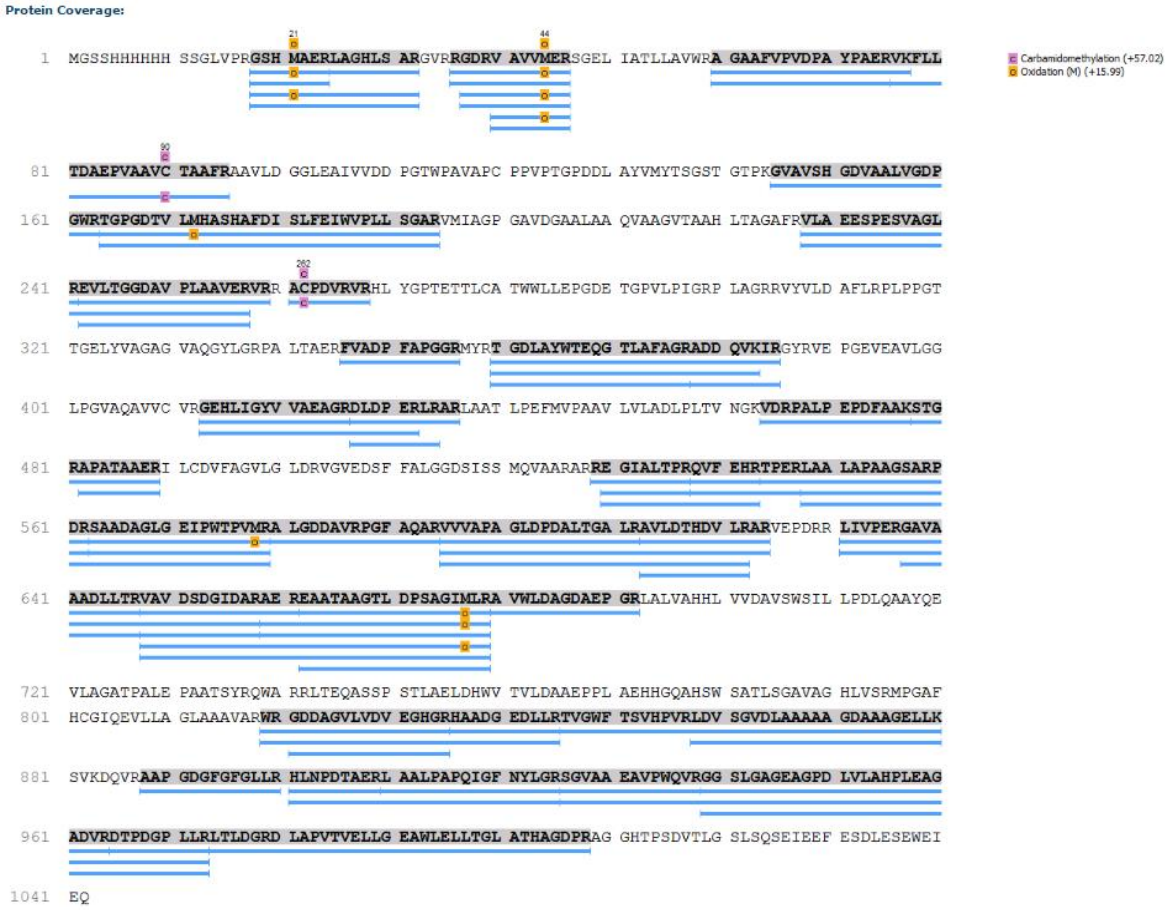


Figure 14. Trypsin digest of Tcp11 (Wild type Tcp11 construct without MBP).

According to the digestion result (figure 14), an improvement coverage was obtained after removing the MBP part in the target protein, with more than 50 % coverage. However, no digested peptide containing the conserved serine residue was detected. Another potential solution to improve the coverage is employ another protease agent. Chymotrypsin is orthogonal to trypsin in specificity, cleaving on the carboxyl site of hydrophobic aromatic amino acids Phe, Trp, Tyr, and Leu (Swiss Institute of Bioinformatics 2010). The combination digestion of chymotrypsin and trypsin may improve additional coverage of the protein (figure 15).

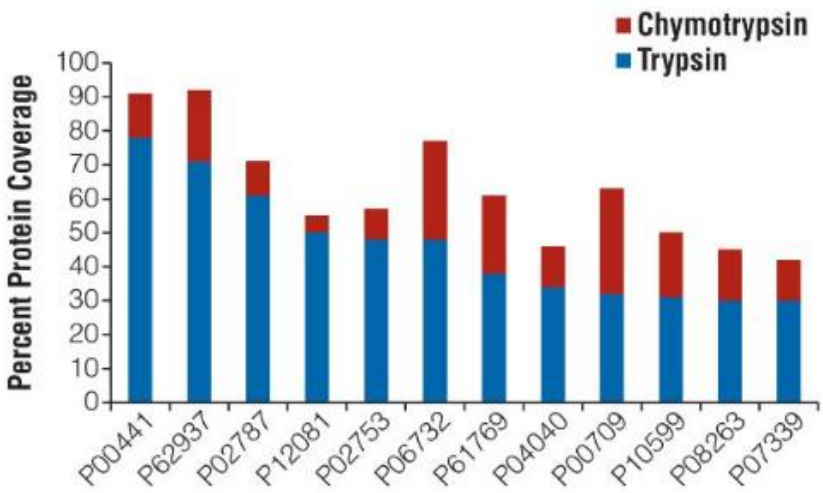


Figure 15. Increase in protein coverage obtained from the use of chymotrypsin and trypsin (Simpson, et al. 2010).

Secondly, different *E.coli* strain (HM0079) can be used instead of BL21. *E.coli* HM0079 is known carrying Ppant transferase gene (*Sfp*) from *Bacillus subtilis* in the chromosome (Gruenewald, et al. 2004). Subsequently, Acyl carrier protein hydrolase (AcpH) gene can be activated. This gene may remove Ppant-arm from the carrier protein (figure 16), by decreasing CoA levels in *E.coli* (Magnuson, et al. 1993).

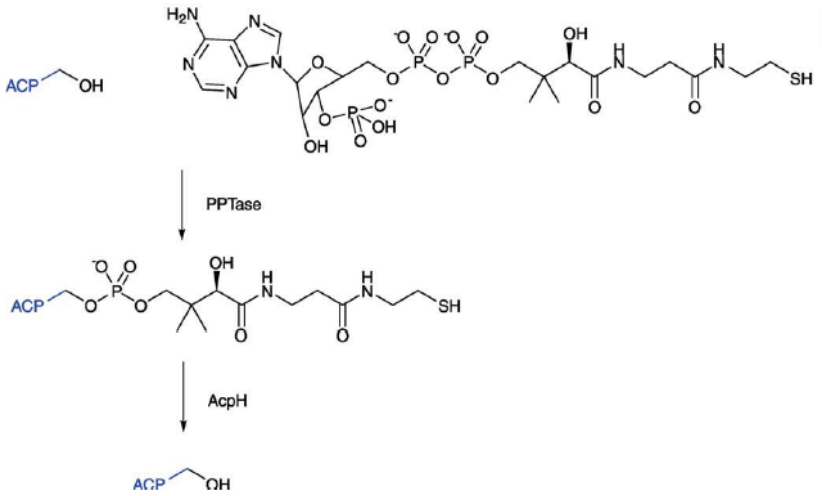


Figure 16. Acp Phosphodiesterase to remove the Ppant-arm (Beld, et al. 2014)

Lastly, the CoA-fluorophore method (figure 17) was performed to detect 4'phosphopantetheinylation with labeled substrate (CoA). CoA 647 is a photostable fluorescent substrate used to label Acp-tag fusion protein exposed on the surface of living cells. The fused protein can be easily detected in SDS-PAGE gel with maximum 660 nm for excitation and 673 nm for emission.

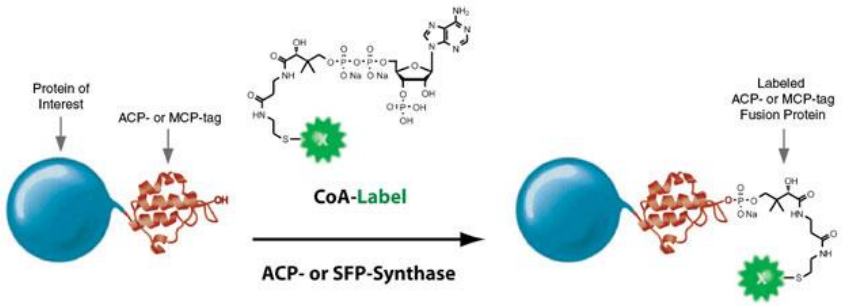


Figure 17. PCP-tag labeling reaction. (NEB 2016)

Regarding to the result, CoA-fluorophore labeling reaction has high efficiency to process the PCP site. Samples were treated with *Sfp* and labeled CoA before it showed by a luminescence band during the 4'-phosphopantetheinylation step. In the figure 19, when the conserved serine residue was mutated (S497A), the labeled Ppant cannot attach to another downstream serine residues displayed by no fluorescence band (column 4 figure 19). Furthermore, the mutation in non-conserved serine residues (S499A and S500A) showed an excessive amount of fluorescent band (figure 20). It revealed that the conserved serine (S497) residue is still active during 4'-phosphopantetheinylation. Meanwhile, a fluorescent band appeared in the triple knockout serine mutations (S497A/S499A/S500A). Thus, probably a mistake was made during operating the assay. Since, the result should be negative, as all of the serine residues were mutated and there are no active sites for Ppant-arm installment.

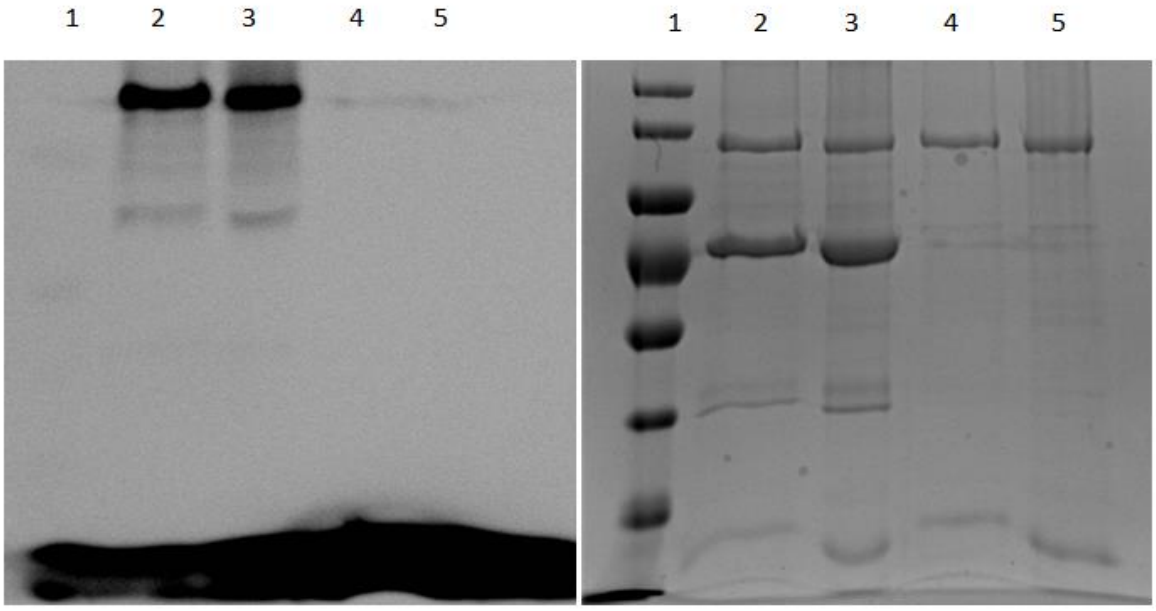


Figure 18. Fluorophore-CoA labeling result (left gel) for Tcp11-ComA construct (lack of MBP). Coomassie blue staining (right gel) to confirm the existence of target protein. Marker (1); Sample treated with *Sfp* (2&3); Sample treated without *Sfp* (4&5).

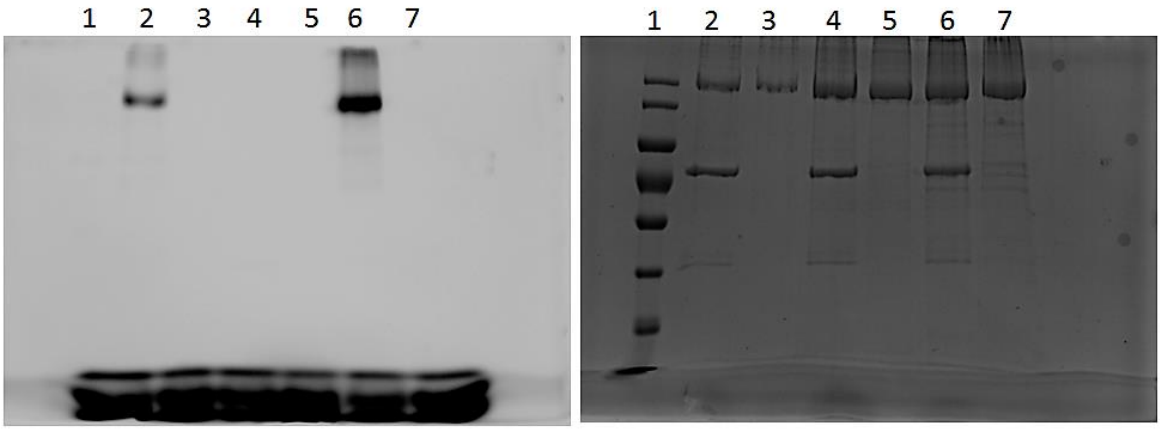


Figure 19. Fluorophore-CoA labeling result (left gel) for Tcp11 constructs (fused with MBP): WT (2&3), S497A (4&5), S497A/S499A/S500A (6&7). Coomassie blue staining (right gel) to confirm the existence of target protein. Marker (1); Samples treated with *Sfp* (2, 4, 6); Samples treated without *Sfp* (3, 5, 7).

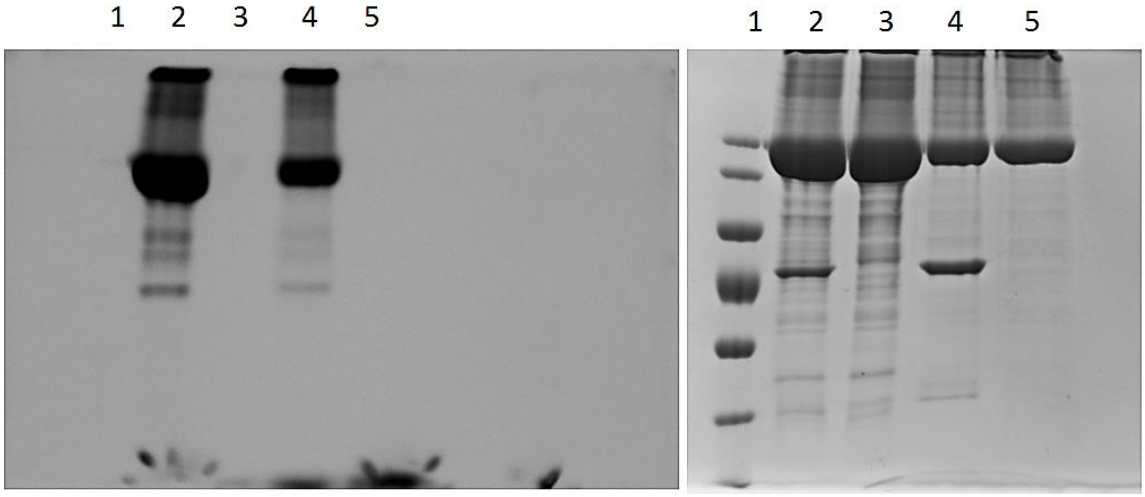


Figure 20. Fluorophore-CoA labeling result (left gel) for Tcp11 mutants construct (fused with MBP): S499A mutant (2&3), S500A (4&5). Coomassie blue staining (right gel) to confirm the existence of target protein. Marker (1); Samples treated with *Sfp* (2, 4); Samples treated without *Sfp* (3, 5).

4. Conclusions

In this project, 4 different mutants were constructed and expressed in *E.coli* in order (S497A; S499A; S500A; S497A/S499A/S500A) to investigate *in vitro* the role of PCP-domain of Tcp11 in Ppant-arm formation. According to the result, the designed mutants were expressed as soluble protein. Pyrophosphate assays showed that all mutants can still perform the adenylation reaction, thus can still activate D/L-Hpg amino acid. In the trypsin digestion assay, probably due to ineffectiveness of the trypsin as a protease agent, no protein digests were detected containing phosphopantetheinyl-arm. The reason cannot be elaborated further, since the trypsin still cut the MBP sequence in the N-terminus. Furthermore, removing the MBP part from the protein has significantly improved the coverage of the peptides sequence. In order to observe the phosphopantetheinylation of Ser497 catalyzed by *Sfp*, a CoA-fluorophore-based method was performed. According to CoA-fluorophore result, the labelled CoA has better efficiency instead of trypsin digestion. The Ser497 seems still taking the crucial role during 4'-phosphopantetheinylation step, confirmed by no obtained band from CoA-fluorophore result. The triple serine knockout proved inactive showing a similar result with the S497A mutant, due to the mistake during operating the assay.

To summarize, studying the conserved serine of thiolation domain in *in vitro* is important to investigate the anchoring Ppant-arm function of the Hpg22 activating module. The Ser497 is crucial for 4'-phosphopantetheinylation of the PCP-domain of Tcp11, whereas the neighboring serine residues (Ser499 and Ser500) are not involved in the process.

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