SEPTEMBER

WEEK 2

Protein Purification (Ni-NTA)

10/09/2021

Equilibration Buffer

Component	Stock Concentration	Working Concentration	Volume for 30 mL
Sodium Phosphate Buffer (pH 7)	0.1 M	50 mM	15 mL
PMSF	1 M	1 mM	30 uL
Sodium Chloride	2 M	0.5 M	3 mL
BME		0.05 %	15 uL
Glycerol		5 %	1.5 mL
Triton X-100	100 %	0.5 %	150 uL
Autoclaved MilliQ	Make upto 50 mL		10.3 mL

Wash Buffer (50 mL)

Component	Stock Concentration	Working Concentration	Volume for 50 mL Wash Buffer
NaPB (pH 7)	1 M	50 mM	2.5 mL
Sodium Chloride	5 M	0.5 mM	5 mL
EDTA	0.5 M	1 mM	100 uL
Autoclaved MilliQ	Make upto 50 mL		

Elution Buffer (50 mL)

Component	Stock Concentration	Working Concentration	Volume for 50 mL Elution Buffer
NaPB (pH 7)	1 M	50 mM	2.5 mL
Sodium Chloride	5 M	0.5M	5 mL
EDTA	0.5 M	1 mM	100 uL
Imidazole	1 M	250 mM	12.5 mL
Autoclaved MilliQ	Make upto 50 mL		

10/09/2021

Ni-NTA Purification

- The flow through (filtrate collected after loading solubilised protein) and wash buffer were collected separately in 50 mL falcon tube.
- The elution flow through was collected in fractions of 1.5ml in 21, 2 mL Eppendorf tubes.

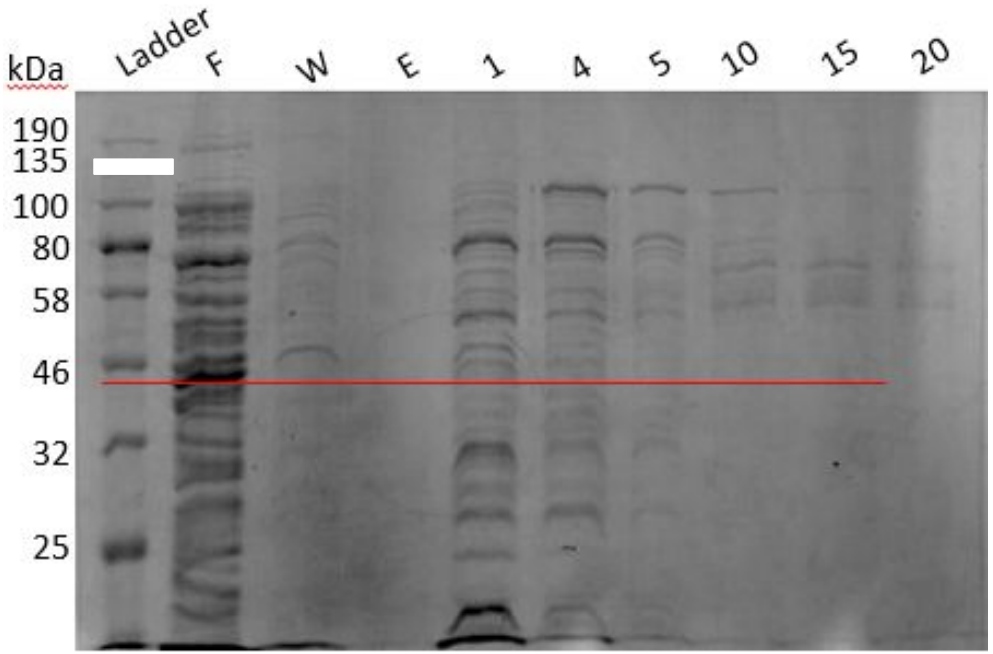
SDS PAGE of Purified Protein

11/09/2021

- The collected fractions were run on SDS along with collected wash through and flow through.
- Stacking Gel : 6 %
- Resolving gel : 13 %

SDS PAGE

Annotations:
F: Flow Through
W: Wash Flow Through
E: Elution Flow Through
1-20: Elution Fractions



Urea Dialysis

1) Inoculation
2) Induction
3) Lysis and sonication
4) Washing the pellet
5) Urea Treatment
6) Ni NTA
7) SDS
8) Dialysis

Inoculation and Induction

- Primary inoculum incubated overnight (10mL) (12hrs).
- Secondary inoculum (1L) incubated till OD600 reached 0.6
- Secondary inoculum induced with 1mM IPTG and incubated at 35 C for 5 hrs.
- Cells pelleted down at 7000 RPM for 20 mins and stored at -20 C.

Lysis and Sonication

14/09/2021

Lysis Buffer

Components	Stock concentration	Working Concentration	Volume required for 20 mL solution
Sodium Phosphate Buffer (pH 7)	1 M	50 mM	1 mL
PMSF	1 M	1 mM	20 uL
Sodium Chloride	5 M	0.5 M	2 mL
BME		0.05 %	10 uL
Glycerol		5 %	1 mL
Triton X-100	100 %	0.5 %	100 uL
Protease Inhibitor	1 tablet(EDTA free)	1 tablet (EDTA free)	1 tablet (EDTA free)
Autoclaved Milli Q	To make up 20 mL		

Sonication

On time	30 secs
Off time	30 secs
Amplitude	55%
Total cycles	15
Total time	15 mins

Sonication carried out in pulse mode

Washing the pellet

Component of **Wash Buffer**	Stock Concentration	Working Concentration	Volume for 50 mL Wash Buffer
NaPB (pH 7)	1 M	50 mM	2.5 mL
Sodium Chloride	5 M	0.5 mM	5 mL
Triton X	100%	0.5%	250 uL
Autoclaved MilliQ	Make upto 50 mL		

1. Wash with wash buffer 20 ml twice.
2. Wash with 20 ml 10 mM CaCl2.

Urea Treatment

Component of **Extraction Buffer**	Stock Concentration	Working Concentration	Volume for 70 mL Extraction Buffer
NaPB (pH 7)	0.1 M	50 mM	3.5 mL
Sodium Chloride	5 M	0.5 mM	7 mL
Urea	10 M	8M	56ml
Autoclaved MilliQ	Make upto 70 mL		

- Resuspend the pellet in 30ml Extraction Buffer.
- Centrifuge it for 1hr 15 min at room temperature 11000rpm.
- Collect the supernatant.

Ni-NTA Purification

- The column is equilibrated with 30ml equilibration buffer.
- The flow through (supernatant) is passed through Ni NTA column and collected in 50ml falcon tube.
- The column is washed with 50 ml wash buffer and wash flow through is collected in 50 ml falcon tube.
- The column is eluted with 50 ml elution.
- The elution flow through was collected in fractions of 1ml in 30, 2mL Eppendorf tubes.

Ni NTA buffers

Component of **Wash Buffer**	Stock Concentration	Working Concentration	Volume for 60 mL Wash Buffer
NaPB (pH 7)	1 M	50 mM	3 mL
Sodium Chloride	5 M	100 mM	1.2 mL
Imidazole	3M	20mM	0.4 ml
Urea	10M	8M	48ml
Autoclaved MilliQ	Make upto 60 mL		

Component of **Elution Buffer**	Stock Concentration	Working Concentration	Volume for 60 mL Elution Buffer
NaPB (pH 7)	1 M	50 mM	3 mL
Sodium Chloride	5 M	100 mM	1.2 mL
Imidazole	3M	400mM	8 ml
Urea	10M	8M	48ml
Autoclaved MilliQ	Make upto 60 mL		

SDS PAGE of Purified Protein

- The collected fractions were run on SDS along with collected wash through and flow through.
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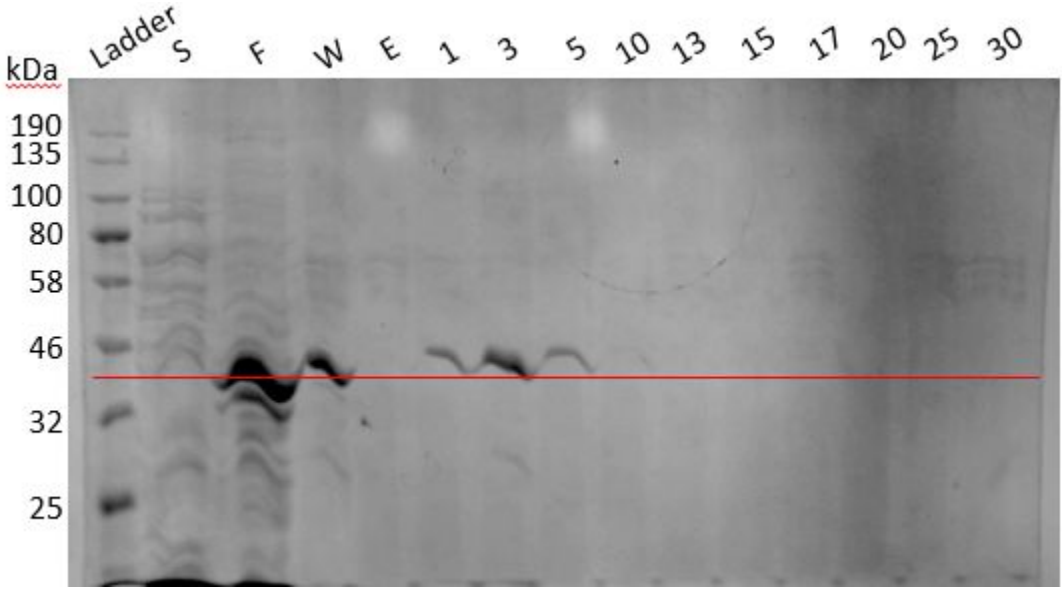

Dialysis

- Do dialysis 4 times for 8-10 hrs: 6M Urea, 4M Urea, 2M Urea, 0M Urea.

Component of **Dialysis Buffer**	Stock Concentration	Working Concentration	Volume for 500 mL
NaPB (pH 7)	1 M	50 mM	25 mL
Sodium Chloride	5 M	150 mM	15 mL
Urea	10M	6M	300 mL
		4M	200 mL
		2M	100 mL
Autoclaved MilliQ	Make upto 500 mL		

Nanodrop (Protein Concentration A280)

The protein concentration was very low. We believe that most of the proteins were precipitated during the purification process. Alternatively, we tried to remove the urea slowly using buffer exchange method using protein concentrator columns. The blank had an error and protein concentration had negative values.

Ni-NTA of Flowthrough solution

- The column is equilibrated with 30ml equilibration buffer.
- The collected flow-through from 14-09-202 was incubated with Ni-NTA slurry for 1 hour at room temperature on a tube rotator.
- The flow through is passed through Ni NTA column and collected in 50ml falcon tube.
- The column is washed with 50 ml wash buffer and wash flow through is collected in 50 ml falcon tube.
- The column is eluted with 50 ml elution.
- The elution flow through was collected in fractions of 1ml in 30, 2mL Eppendorf tubes.

Ni NTA buffers

Component of **Wash Buffer**	Stock Concentration	Working Concentration	Volume for 60 mL Wash Buffer
NaPB (pH 7)	1 M	50 mM	3 mL
Sodium Chloride	5 M	100 mM	1.2 mL
Imidazole	3M	20mM	0.4 ml
Urea	10M	8M	48ml
Autoclaved MilliQ	Make upto 60 mL		

Component of **Elution Buffer**	Stock Concentration	Working Concentration	Volume for 60 mL Elution Buffer
NaPB (pH 7)	1 M	50 mM	3 mL
Sodium Chloride	5 M	100 mM	1.2 mL
Imidazole	3M	400mM	8 ml
Urea	10M	8M	48ml
Autoclaved MilliQ	Make upto 60 mL		

15/09/2021

SDS PAGE of Purified Protein

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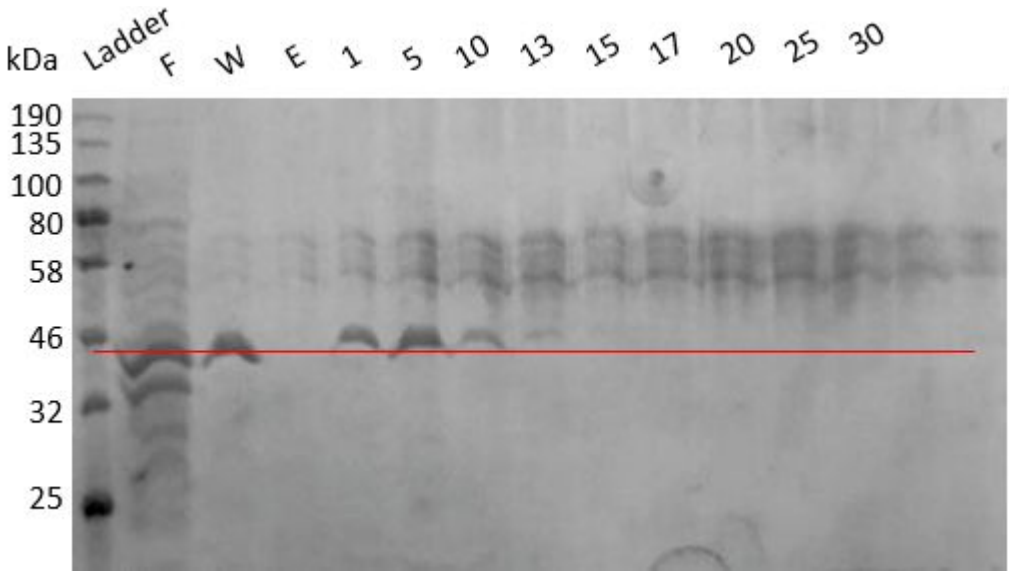

Buffer Exchange Method (Protein Purification)

Number of Washes	Initial Urea Conc. (M)	Initial Solution Volume (mL)	Final Urea Conc. (M)	Final Solution Volume (mL)
1	8	2.5	6	3.33
2	6	2.4	4	3.60
3	4	2.5	3	3.33
4	3	2.1	1.5	4.2
5	1.5	2	0.75	4
6	0.75	2.5	0.45	4.17
7	0.45	2.5	0.18	5.00

Nanodrop (Protein Concentration A280)

The protein concentration was still coming very low. We believe that most of the proteins were precipitated during the purification process.
So, we decided to do **antifungal assay with crude protein.**