Measuring Elasticity of Force sensing proteins using Single Molecule Force Spectroscopy

Angel D S

A dissertation submitted for the partial fulfillment of BS-MS dual degree in Science

Indian Institute of Science Education and Research, Mohali
April 2017

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Certificate of Examination

This is to certify that the dissertation titled "**Measuring elasticity of Force sensing proteins using Single Molecule Force Spectroscopy**" submitted by **Angel D S** (Reg.No MS12105) for the partial fulfillment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Arjit Kumar De

Dr. Ujjal Gautam

Dr. Sabyasachi Rakshit
(Supervisor)

Declaration

The work presented in the dissertation has been carried out by me under the guidance of Dr. Sabyasachi Rakshit at the Indian Institute of Science Education and Research, Mohali. The work has not been submitted in part or in full for a degree, a diploma or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgment of collaborative research and discussions. This thesis is a bonifide record of original work done by me and all sources listed within have been detailed in the bibliography.

Angel D S
(Candidate)

Dated:

In my capacity as the supervisor of the candidate's project work, I certify that the above statements are true to the best of my knowledge.

Dr. Sabyasachi Rakshit
(Supervisor)

Acknowledgements

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I would like to thank my parents, grandmother and brothers for their constant support, encouragement, care and prayers. I am so grateful for all the love and care I received from Dr. Lini Mathew, Mathew Alexander, Raju Chacko, Mini Chacko, Babu Rao, Molly Babu and all the family members. I will always cherish all the beautiful moments I spent with you.

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Abstract

Mechanotransduction is one of the major processes that facilitate hearing. Proteins that are involved in mechanotransduction are known as tip-links and mutations in these proteins cause hearing-loss. We plan to understand how mutations alter the mechanical stability of the proteins using single molecule force spectroscopy using Atomic Force Microscopy. To do, we first developed an enzymatic stapling protocol to make a polyprotein of I27 and sandwiched our protein of interests. In this thesis work, I prepared the constructs of I27 and the terminal extracellular domain of Cadherin-23 (a tip-link protein) and its mutant which can be used for molecular stapling.

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CHAPTER 1

INTRODUCTION

Mechanical elasticity of proteins play versatile role in regulating cellular processes. Our hearing is one of such processes where the elasticity of proteins forming tip-links in hair-cell stereocilia are tightly regulated by mechanical stimuli. Mutational modifications on these proteins cause hearing loss. Here we aim to measure the mechanical stability of the tip-links proteins and understand how mutations alter their stability. We use single molecule force spectroscopy for the study using Atomic force Microscope (AFM).

Over the last three decades AFM has established to be a valuable experimentation technique to probe the structure and energetic of a protein. The standard protocol to study the elasticity of proteins using force spectroscopy at the single molecule level is to use a polyprotein construct. This is to identify specific events over identical features coming from non-specific molecules. A polyprotein is mainly made of repeated units of a reference protein where protein under probe is sandwiched. Since the elasticity of theI27 protein is well-studied, I27 is widely used as reference. I27 is a 27th domain of human titin protein, which is the largest protein in the human body.

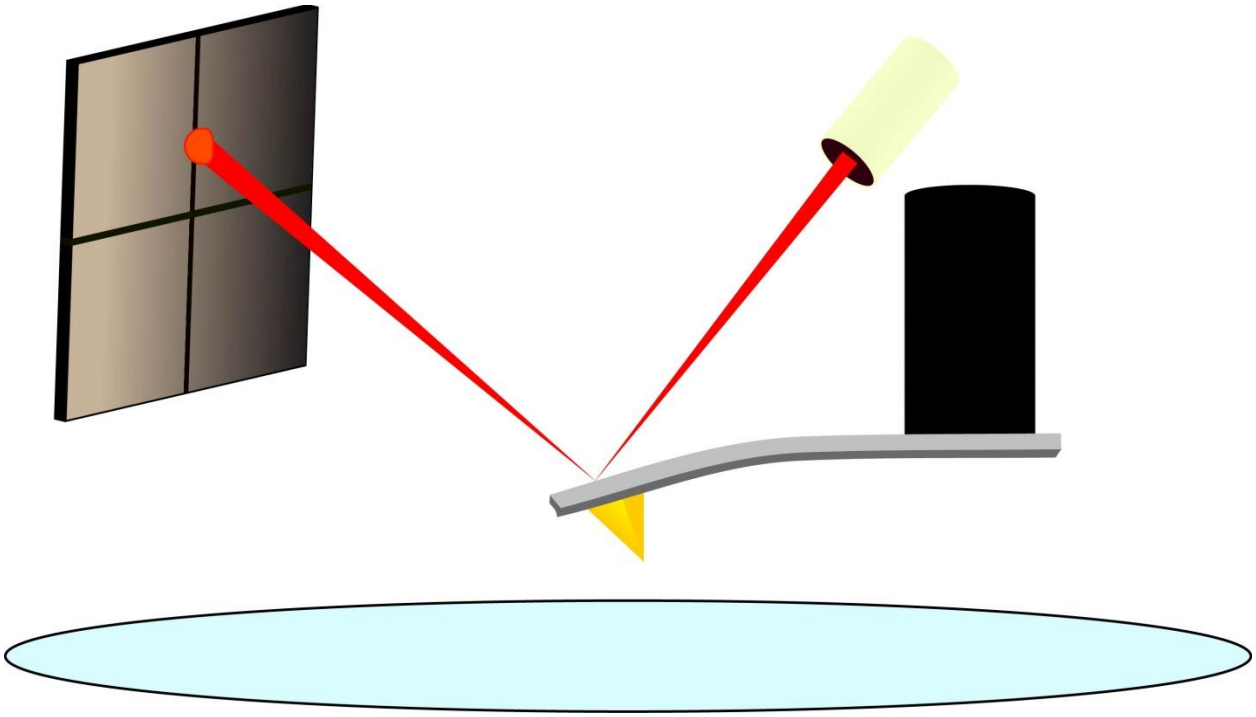


Figure 1: Shows the illustration of AFM

In force-spectroscopy, a clean and soft AFM (spring constant~100 pN/nm) cantilever is used for the unfolding of polyproteins.The typical signature of the unfolding of a polyprotein is a saw-toothed pattern in the force *vs* distance curve which is best described by Worm-like chain (WLC) polymer model. The area under each unfolding force-curve measures the entropic energy of folding. The force-distance feature in force-spectroscopy is dependent on the intrinsic folding of a protein. The rigidity of structure determines the strength of unfolding. It is understood and

studied that topology of the protein plays an important role in unfolding force. Since the secondary structure determines the entire topology of the protein, understanding the unfolding strength of these folds is important. For example, β- sheets offer much resistance than α-helix and random coil. Single molecule studies have the added advantage of exposal of rare events, which cannot be observed during the ensemble averaging of events by chemical denaturation.

Engineering polyproteins has always been a challenge for experimentalists. Generic methods like cloning have been under use since long, which takes time and labor. To tackle this, we have conceived a new idea to design a polyprotein by enzymatic stapling process. This process needs the recombinant modification of each unit in the polyprotein separately, and then stapling them together at any sequence and any number of repeats using sortagging.

Cadherin-23(Cdh23) is one of the tip-links proteins that response to mechanical force during hearing and transduce into electrical signal. We therefore, aim to understand the mechanical elasticity of Cdh23. We designed a polyprotein of the terminal extracellular domain (EC1) of Cdh23using enzymatic stapling (Methods). Since, domains unfold stochastically under force it was not easy to study the behavior of EC1(first domain) of Cadherin23 alone. As a result we were unable to get fingerprint of EC1 domain of Cdh23 protein using this method. We plan to develop a polyprotein (8 repeats) of I27 and sandwichCdh23 in the middle.

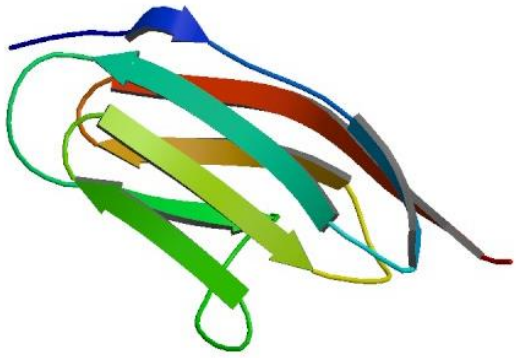


Figure 2: *Representative structure of I27 protein.*

Hypothesis:

Molecular elasticity of tip-links controls the opening of ion-channels in hair-cells during hearing sound of varying intensities.

Objectives:

1. Engineer polyprotein using I27 repeats and incorporate a single Cdh23 (EC1) between I27 repeats.
2. Measure the molecular elasticity of Cdh23-EC1 and its mutants.

CHAPTER 2

MATERIALS AND METHODS

Engineering of I27 protein domain and purification. Constructed two types of I27 domain of Titin protein with His tag was cloned in pET23a(+) vector with primers containing TEV sequence and His tag

2,1 Protocol for PCR [*Polymerase Chain Reaction*]

Components for PCR:

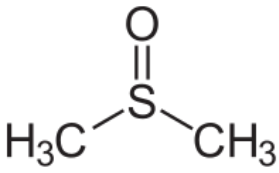
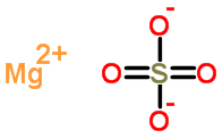
1. Template
2. Enzyme
3. Buffer
4. DMSO (optional)
5. dNTP
6. Forward Primer
7. Reverse Primer
8. Mg2+ (optional)
9. Double Distilled water

Template: Piece of DNA which we are going to amplify (DNA of our interest)
Primers: Piece of DNA which initiates the chain reaction.
Enzyme: Commonly called 'Polymerase enzyme' which helps in binding of our "Primers" to "Template".
Buffer: Maintains the pH of the solution.
dNTP: Provides the nucleotides for the new forming strands.
DMSO: Helps to reduce the temperature of the annealing and also breaks the internal loop which forms.
Mg2+: Enhances the functioning of polymerase enzyme and also helps in the binding of *Primer* to *Template*.

1. Prepare the master mix.
2. Set the Melting, Annealing, Extension temperatures and number of cycles.

Chemicals Used:

Table 2.1:

Function	Structure	Chemical name	
Required to reduce the melting temperature of primers by inhibiting GC interaction		Dimethyl sulphoxide	DMSO
Helps in primer annealing and act as catalyst for polymerase		Magnesium sulfate	MgS04

2,2 DNA Gel Electrophoresis

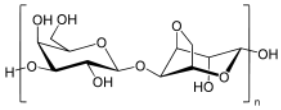
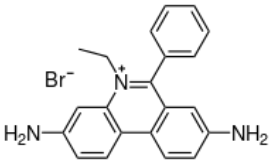
Electrophoresis is used as a method to separate DNA based on their movement under the influence of Electric current. It's used for two different purposes a) To determine the presence and size of DNA. b) Purification of the band (DNA of our size) of our interest.

1. Weigh the Agarose and make 1 percent solution in TAE.
2. When the solution is cooled down (around 45C) add EtBr to it. At room temperature Agarose will solidify.
3. Pour it in gel casting chamber and let it solidify.

Chemical used:

Table 2.2:

Function	Structure	Chemical name	

Cross linked structure will help in separation of bands		Polysaccharide made of disaccharide	Agarose
Fluorescent tag for DNA, it intercalates with DNA [absorbs @ 210 nm and emits @605 nm]		Ethidium bromide	EtBr

2.3 Double digestion

Digesting/Cutting the construct with the restriction enzymes at both the ends that's why it's double digestion.

1. Take the Template to be digested in a MCT.
2. Add both the Restriction Enzymes to it [Here we used Fast Digest Enzymes and buffers]
3. Keep it at 37^0 C for 30 minutes.

2.4 Ligation

Fusing of the construct we made to the plasmid (pET23a)

1. Take the Plasmid to which we are going to clone and add to it the Insert in the ratio of 3:1.
2. Keep the ligation mixture at 25^0 C for 2 hours.

2.5 Transformation

The heritable modification of the properties of a competent bacterium by DNA from another bacterial strain.

1. Thaw the competent cells and plasmid in ice.
2. Switch on the water bath and set in 42°C.
3. Take 50 ng - 100 ng of plasmid and transform it in competent cells.
4. Keep

2.6Colony PCR

Finding the positive colony using PCR.

2.7 Primary Culture

Take the positive colony in a 5/10mL LB media and add antibiotic to it and incubate the culture in 37ºC for 14-16 hours with shaking.

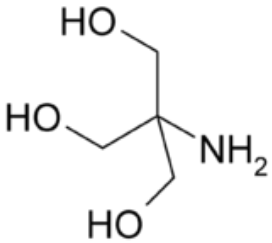
2.8 Plasmid Isolation

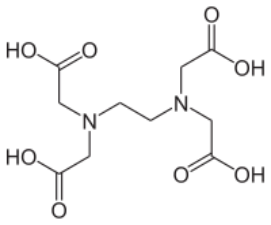
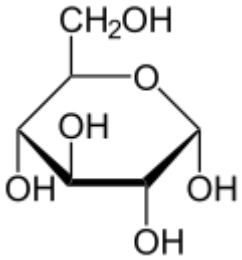
Take the grown culture and do plasmid isolation. Here our plasmids are inside the competent cells.

1. Centrifuge around 2 mL of culture and throw the supernatant.
2. Now our cells are in pellet, make sure the pellets are dry.
3. Add Resuspension buffer and resuspend the pellet in this buffer with rigorous shaking.
4. Add lysis buffer which breaks the cells open and DNA comes out shake gently 5-6 times.
5. Add neutralization buffer and gently invert it 5-6 times.
6. Pass it through the column and centrifuge it, now all the debris will go down through the column and only DNA gets bound to the column.
7. Now remove the flow through and wash the column with wash buffer 1, this will wash away all the salts.
8. Now again wash it with wash buffer 2 this will wash away all the RNAse and proteins.
9. Now take a new fresh MCT and label it, keep the column in the new MCT and add minimum amount of Elution buffer or molecular biology water(30µL).

Buffers used:

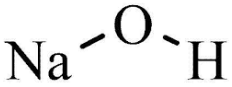
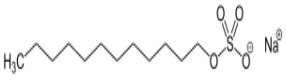
Table 2.3:
Resuspension buffer:

Function	Structure	Chemical name	
Maintain pH @ 7.5		tris(hydroxymethyl)aminomethane	Tris

Sequestering metal ions via chelation.		Ethylenediaminetetraacetic acid	EDTA
Maintains the osmotic pressure inside cell so that cells don't burst.		$C_6H_{12}O_6$	Glucose
To degrade cellular RNA when cells are lysed.		Ribonuclease	RNase

Lysis buffer:

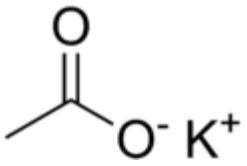
Table 2.4:

Function	Structure	Chemical name	
Helps to break down the cell wall and also disrupts the hydrogen bonding between the double helix of DNA converting to ssDNA		Sodium hydroxide	NaOH
Solubilize the cell membrane and denatures the protein which helps in the separation of protein from plasmid.		Sodium dodecyl sulphate	SDS

Neutralization buffer:

Table 2.5:

Function	Structure	Chemical name	

Decreases the alkalinity of the solution and hydrogen bonds re-establishes.		Potassium acetate	CH_3COOK

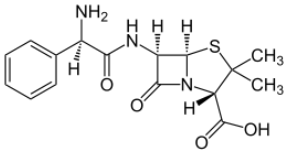
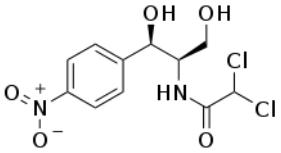
2.9 Transformation in Expression Strain:

Primary culture:

1. Choose one colony from the plate and grow the colony in a selection marked antibiotics in my case Ampicillin and Chloramphenicol, keep the 10mL culture in 37C with shaking for 14-16 hrs.

Chemicals used:

Table 2.6:

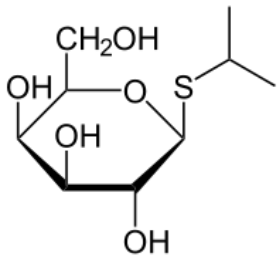
Function	Structure	Chemical name	
Act as selectable marker.		Ampicillin	Amp
Helps in selection of our interested vector.		Chloramphenicol	Chl

Secondary culture:

1. Make 1 Litre LB media and autoclave it.
2. Add antibiotics markers.
3. Add primary culture to the final concentration of 1% in 1 liter secondary culture.
4. Keep the secondary culture at 37°C in incubator with shaking until the OD reaches 0.4-0.6.
5. Once OD reaches 0.4-0.6, induce culture with IPTG(1mM final concentration) and keep it back in the incubator for another 5-7 hrs.

Chemicals used:

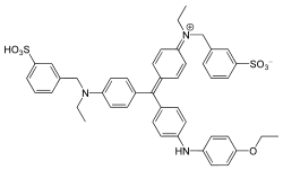
Table 2.7:

Function	Structure	Chemical name	
Helps in triggering the transcription. (DNA to mRNA)		Isopropyl β-D-1-thiogalactopyranoside	IPTG

2.10: Checking for the over expression of desired protein:

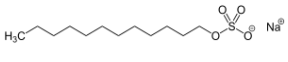
1. Made one SDS-PAGE and after taking 1ml culture of induced and uninduced cells pelleted them down.
2. Then added sample loaded dye and heated for 5 minutes at 99C
3. Centrifuged for 10 min at 13000 rpm to remove all cell debris.
4. Ran the supernatant on SDS-PAGE
5. After completion stained the gel with coomassie blue and looked for the over expression.

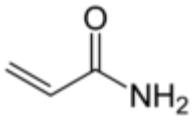
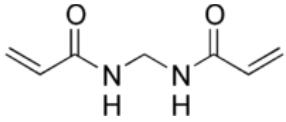
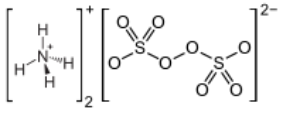
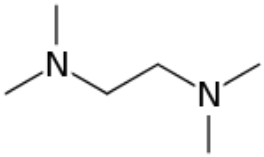
Table 2.8:

Function	Structure	Chemical name	
It binds with protein through ionic interactions		triphenylmethane	Coomassie blue

Chemical used:

Table 2.9:

Function	Structure	Chemical name	
Denatures protein by breaking non covalent bond.		Sodium dodecyl sulphate	SDS

Separation of molecules depending on charge.		prop-2-enamide	Acrylamide
Helps in crosslinking between two linear acrylamide planes		N,N-Methylenebisacrylamide	Bisacrylamide
Oxidizing agent which helps in continuing the polymerization reaction..		Ammonium persulphate	APS
Initiates the polymerization		Tetramethylethylenediamine	TEMED

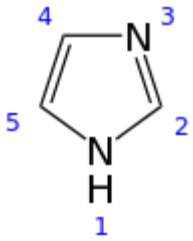
2.11:Sonication:

1. Sonicated the whole 1Litre culture after pelleting cells down in PBS with 10mM Imidazole.
2. After sonication pelleted the debris down by high speed centrifugation 13000 rpm for 40 min at 4 C.
3. Took supernatant and loaded it on Ni-NTA column.

Chemicals used:

Table 2.10:

Function	Structure	Chemical name	
Helps in maintaining the pH equivalent to human body		Phosphate buffered saline= NaCl +KCl + Na_2HPO_4 + KH_2PO_4	PBS

By competing with Ni-NTA it helps in unbinding of proteins.		$C_3N_2H_4$	Imidazole

2.12: Affinity chromatography using Ni-NTA for purification:

1. Equilibration of column was done with PBS containing 10mM Imidazole
2. Sample was loaded and allowed to pass through Ni-NTA beads in column under gravity.
3. Column was washed with PBS containing 20mM imidazole to remove unwanted proteins
4. Finally elution was done with PBS containing 250 and 500mM Imidazole
5. Ran gel to check the purity of my protein in elution fractions.

2.13 Native gel check for possible dimer:

Same protocol except SDS we add everything to the gel

Table 2.11:

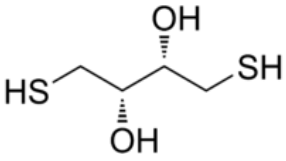
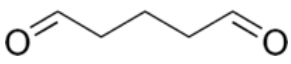
Function	Structure	Chemical name	
Helps in reduction of disulphide bond in protein to -SH		dithiothreitol	DTT

Table 2.12:

Function	Structure	Formula	
Cross links between two proteins.		$CH_2(CH_2CHO)_2$	Glutaraldehyde

2.14 Ligation Independent Cloning:

Primer Design: The site to be mutated should be at the centre of primer leaving 12-15 bp from left and right side.

Template: The plasmid should be expressed in DH5 α strain.

- Made the master mix by adding the template of interest (Plasmid/DNA) to be mutated, primers, DNTps, buffer, enzyme and water.
- Mixture is then placed in the PCR machine.
- Adjusted the annealing temperature accordingly and set the extension time depending on the length of template.
- Checked for PCR amplification in gel electrophoresis.
- Purified the PCR product through PCR cleaner kit.
- Digested the Template DNA using DPN1 restriction enzyme, here we kept one control to check the completion of DPN1 restriction enzyme at 37◦C for 1 hr.
- Table 2.13:

Experiment	Control	
15 ml	1 μL	Template
0 μL	14 μL	Water
0.2 μL	0.2 μL	DPN1

- Did heat inactivation to inactivate the DPN1 enzyme from further interaction for 20 mins at 80◦C.
- Transformed both the control and experimental plasmids in DH5 α cloning vector with Ampicillin resistance and kept it in incubator for 14 hrs at 37°C.
- We got zero colonies in control and many colonies in experiment.[must]
- Took 3 to 4 colonies and send for sequencing.

2.15: Schematics showing synthesis of Polyprotein using Enzymatic Stapling:

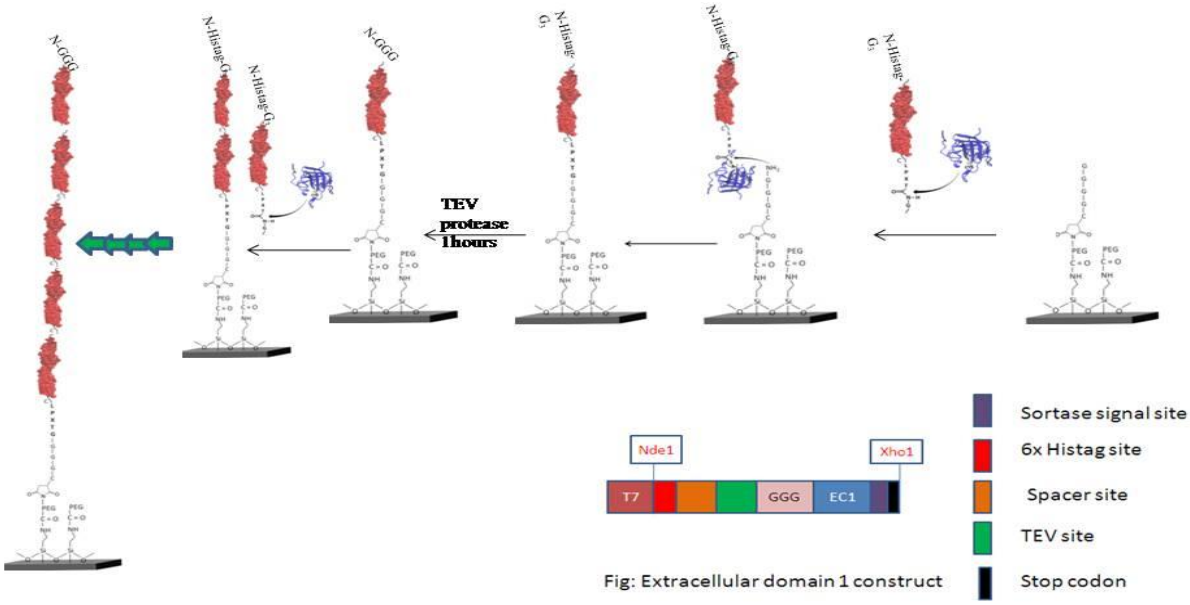


Fig: Extracellular domain 1 construct

Fig.: schematic diagram of polyprotein construction

Figure 3

- Modified the surface and exposed the Glycine (GGG) on surface.
- Made protein and sortase in the ratio of 1:1.25 µM.
- Added protein sortase mixture to the glass slide and incubated for 1 hr.
- Washed the surface for 3 times with calcium buffer to remove excess protein and sortase.
- Added TEV protease and incubated for 1 hr.
- Washed the surface with calcium buffer 3 times to remove the TEV protease.
- Repeated the process as many times to get as many layers we needed.

2.16 Total Internal Reflection Fluorescence Microscopy:

- Modified the surface of coverslip.
- Added sortase and protein in the ratio of 1:1.25 and incubated for 1 hr.
- Washed the surface with calcium buffer for 3 times.

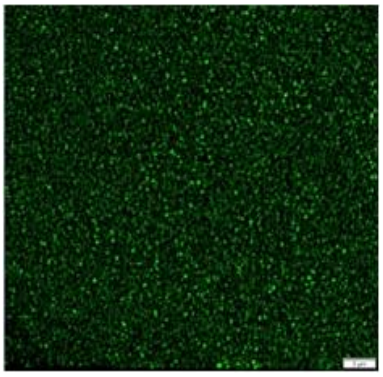
- Added primary antibody (antihis-antibody) and incubated for 1 hr.
- Washed the coverslip with calcium buffer for 3 times.
- Added secondary antibody and incubated for 1 hr.
- Kept the coverslip on the TIRFM and recorded the image, here we got fluorescence.
- 

Figure 4: Fluorescence shown by the attachment of I27 on the surface of coverslip.

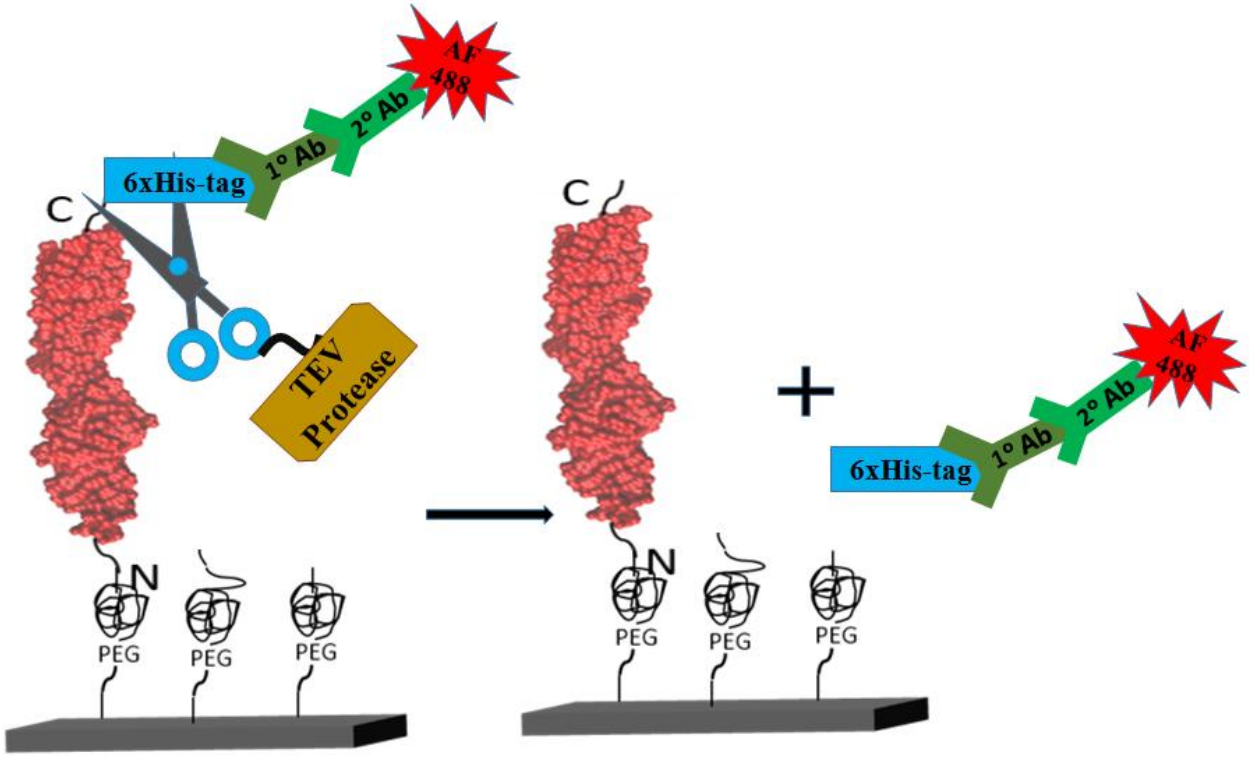
- Added TEV protease which cuts at the site TEV and exposes the GGG site.
- 

Figure 5:[Illustrated by Jagadesh Hazra] Shows the TEV protease cutting at the TEV site and exposing the GGG site for the next layer formation.

- Recorded the TIRFM image after washing with buffer for 3 times.

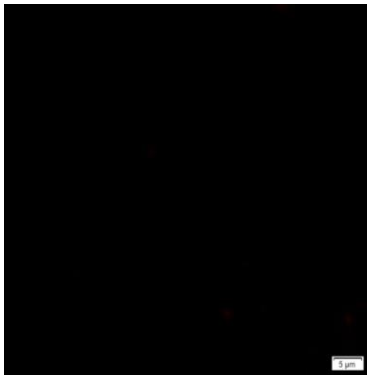
- 

Figure 6: Shows the image after cutting with TEV protease.

- Repeated the experiment four times to make 5 layers.

CHAPTER 3

RESULTS AND DISCUSSION

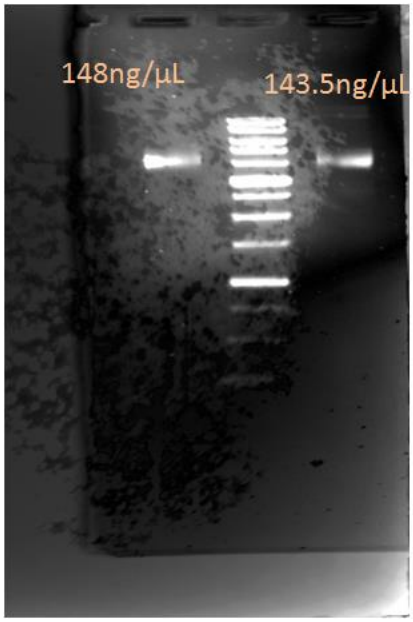
We obtained I27 construct in pQE80L. We designed the primers depending upon the constructs we wanted to make.

The Constructs required are:

1. **Nde1-His tag-Spacer-TEV-3xG-I27-Sort tag-stop-Xho1**
2. **Nde1-His tag-Spacer-TEV-Cysteine-I27-Sort tag-stop-Xho1**

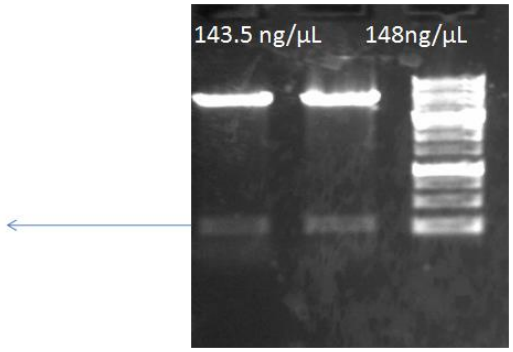
DNA quantification:

Here we ran the plasmid containing our gene of interest to quantify the amount of DNA.



~100ng/µL

Double digestion check:



Arrow shows band of our interest which is I27 at 267 bp.

Table 3.1:

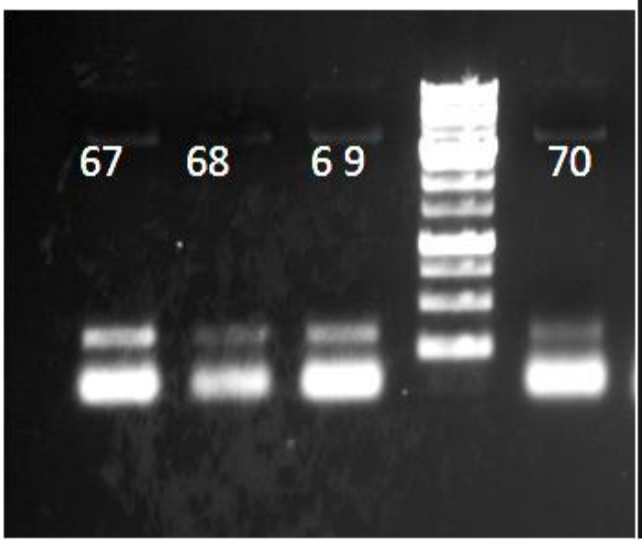
Fast digest BamH1	Restriction enzyme 1

Fast digest Bgl11	Restriction enzyme 2
Fast digest green buffer	Buffer
37°C in water bath	Temperature

Step 1 PCR

- For 3XG_I27
  1. Forward primer: GAAAATCTGTACTTCCAGGGCGGAGGCGGGCTAATAGAAGTGG
  2. Reverse Primer:ATT ATA CTC GAG TCA TTATGA GGA GCC CGT TTC CGG CAG CAA TTC TTT CAC
- For Cys_I27
  1. Forward primer:CACCGAAAATCTGTACTTCCAGGGCTGTCTAATAGAAG
  2. Reverse Primer:ATT ATA CTC GAG TCA TTATGA GGA GCC CGT TTC CGG CAG CAA TTC TTT CAC

Image on the gel after this step



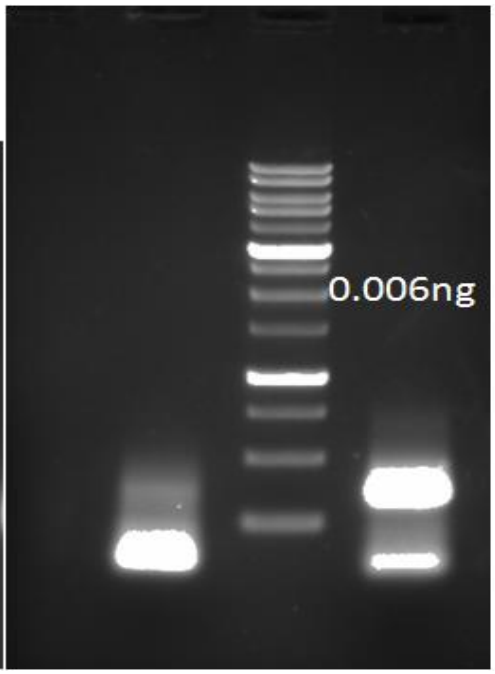


Figure 1 Figure 2

Figure 1: These small bands show the band of our interest labeled with different temperatures.

Figure 2: Shows the bands with varying concentration of template used.

label each lane with marker and draw arrow to point out your product.

Step 2 PCR:

- For 3XG_I27
    1. Forward primer: CATATGCACCATCATCATCATCAT GACTACGACATTCCGACCACC GAAAACCTGTACTTCCAG
    2. Reverse primer: ATT ATA CTC GAG TCA TTATGA GGA GCC CGT TTC CGG CAG CAA TTC TTT CAC
- For Cys_I27
    1. Forward primer: CATATGCACCATCATCATCATCAT GACTACGACATTCCGACCACC GAAAACCTGTACTTCCAG
    2. Reverse primer: ATT ATA CTC GAG TCA TTATGA GGA GCC CGT TTC CGG CAG CAA TTC TTT CAC

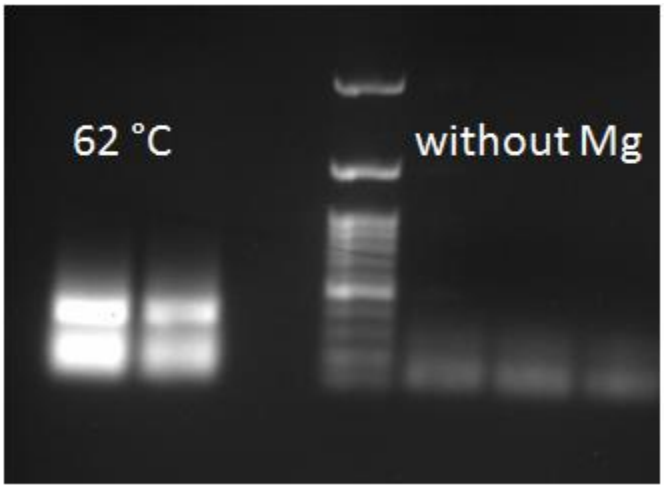


Figure shows the difference in intensities of constructs with and without magnesium.

Step 3 PCR:

- For 3XG_I27
    1. Forward primer: 5:GGA TAT CAT ATG CAC CAT CATCATCAT CATG : 3

Tm = 55.8

    2. Reverse primer: 5:GGA TAT CTC GAG TCA TTA TGA GGA GCC CG:3

Tm = 57.7

- For Cys_I27
    1. Forward primer: 5:GGA TAT CAT ATG CAC CAT CATCATCAT CATG : 3

Tm = 55.8

    2. Reverse primer:5:GGA TAT CTC GAG TCA TTA TGA GGA GCC CG:3

Tm = 57.7

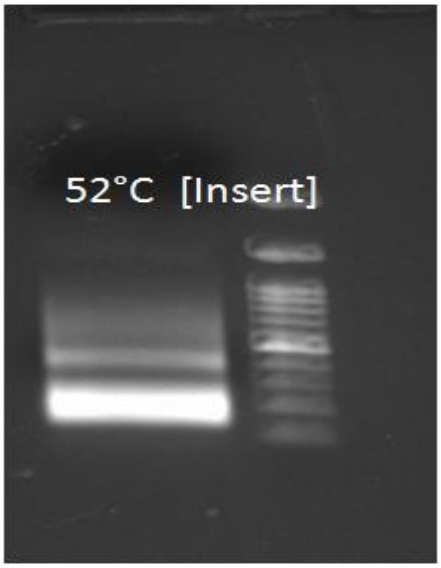


Figure a) Ladder 100 bp and insert is 3XG_I27

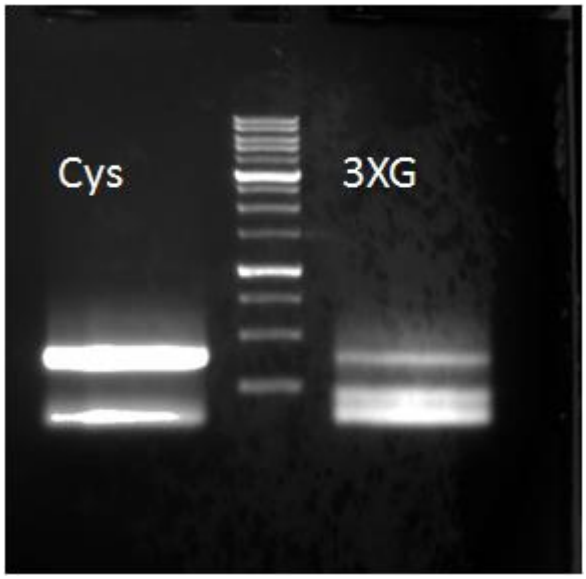


Figure b) Ladder 1 kb [Total size of band was 385 bp]

Cloning:

- Did double digestion of Insert.
- Purified the double digested insert using PCR cleaner kit.
- Double digestion of pET23a vector:

Bright band shows the double digested vector pET23a.

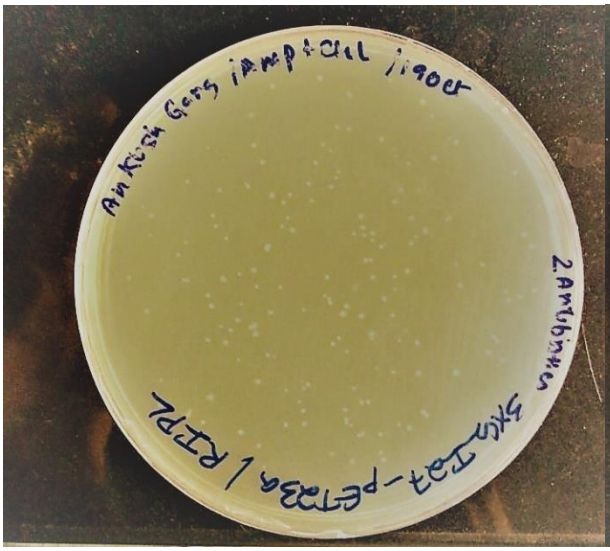
Table 3.2:

Fast digest green buffer	Buffer
0.7 µL	Xho1

0.7 µL	Nde1

Transformation

- Cloning of 3XG_I27 and Cys_I27 in pET23a Vector.
- Done transformation in XL1 Blue competent cells.
- Screened the colonies for the positive colony.
- Made the primary culture from the positive colony.
- Done plasmid isolation with miniprep kit.
- Sent for sequencing the result for Cys_I27.
- Taken 0.7ng of plasmid and done transformation in BL21_RIPL.



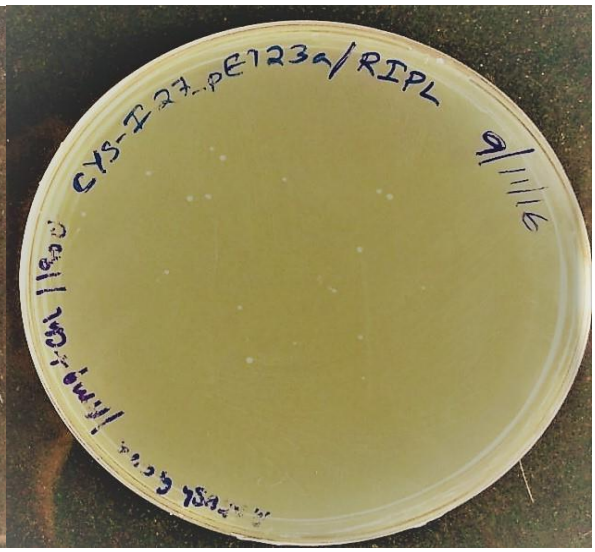


Figure 7a) Figure7 b)

These are expression host for pET23a vector

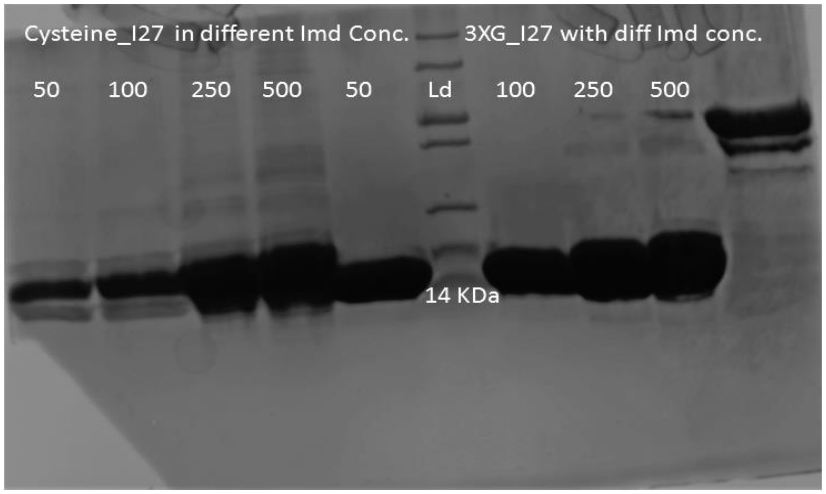
Figure a) is the colonies we got from retransformation of pET23a of 3XG_I27 in BL21_RIPL

Figure b) is the colonies we got from retransformation of pET23a of Cys_I27 in BL21_RIPL.

Purification:

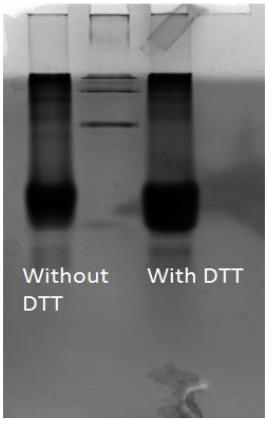
- After this we took one colony from each and grown in primary culture (LB media with ampicillin and chloramphenicol).
- Kept the solution in 37 C for 14-16 hrs in incubator with shaking.
- Then we added 1% of secondary culture from primary culture.

- Kept the solution for incubation at 37 C in incubator until the OD reached around 0.4-0.6 OD.
- On reaching 0.4 we induced secondary culture with IPTG.
- Then kept the solution for another 5 hrs in the incubator at 37 C with shaking.
- Centrifuged the whole solution to pellet the cells.
- Then added Lysis buffer which in my case PBS buffer with 10mM imidazole.
- To it we added Lysozyme and
- Kept each of the solution for sonication for 30 minutes.
- After sonication we centrifuged the solution to remove the cell debris pellet and collected the supernatant.
- After this I purified the protein using Ni-NTA in native form.
- After this we ran an SDS-PAGE to check the expression of our proteins.



This figure shows a SDS-PAGE with different concentrations of Imidazole used for the wash and elution of protein Cys_I27 and 3XG_I27. In this we got our bands around 14 KDa this may be due to anomalous mobility of protein in SDS-PAGE. Our protein of interest should come around 13.2 KDa.

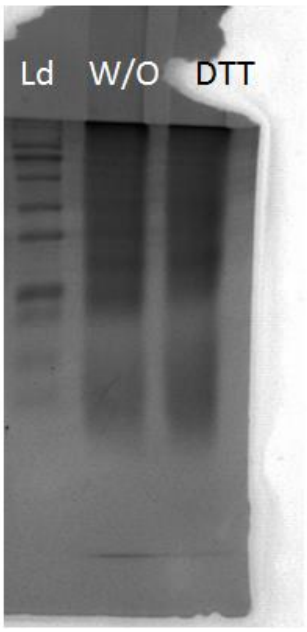
- After this we tested for any possible dimer formation, for this we ran a native page gel one with DTT and other without DTT only for Cys_I27



Here we got only one band in both control and experiment this suggests that there are two possibilities a) there is no dimer b) the concentration of dimer is too low or under detectable levels.

Crosslinking with glutaraldehyde:

- Native gel alone cannot verify the dimer presence so to conform we tried a simple experiment with crosslinker molecule which is glutaraldehyde so we ran an SDS-PAGE with 0.1% glutaraldehyde for cys_I27 with DTT and without DTT



The role of glutaraldehyde was to crosslink the polymer when it is used in its optimum quantity. If the concentration increases, it can lead to the formation of multimeric structures of proteins. Here my gel picture suggests that the concentration of glutaraldehyde was high which made part

of protein's hydrodynamic volume so big and inhibited in entering the gel that's why there is band in the well, and it is smeared cause of all possible multimers got produced.

Purification of EC1 GGG

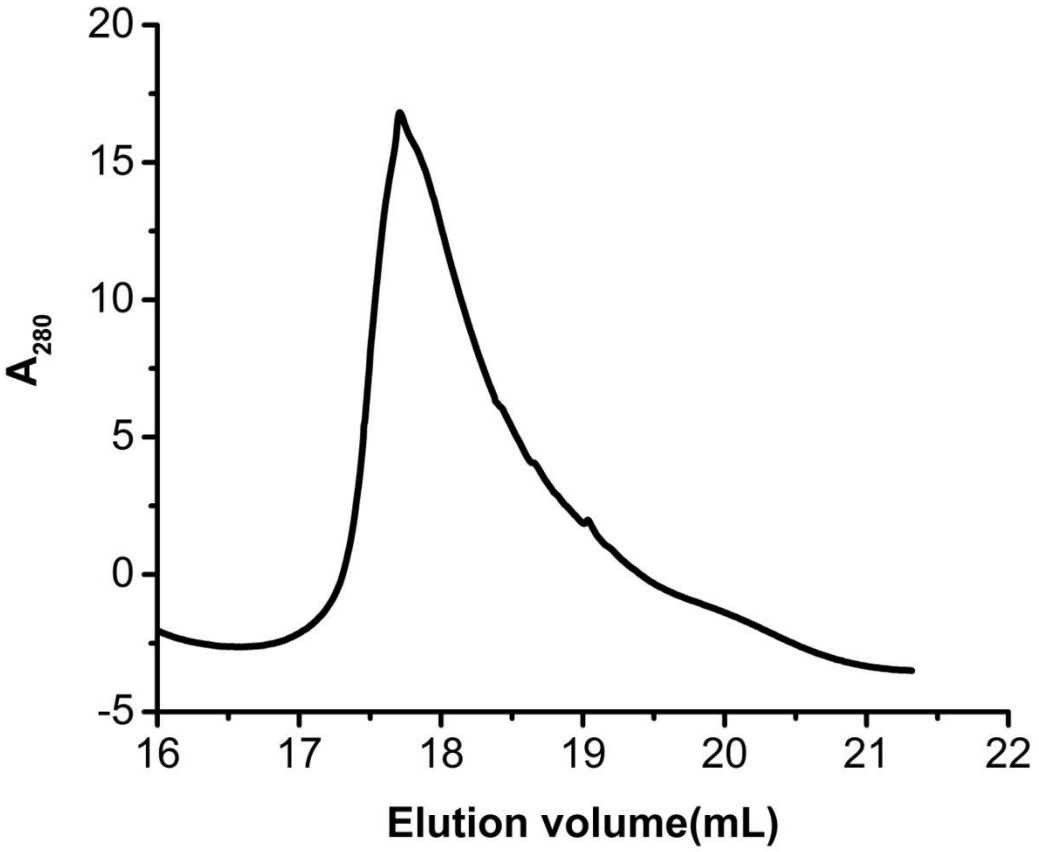


Figure 9: Showing the Size exclusion chromatography of EC1 GGG

Atomic Force Microscopy

We pulled the polyprotein with velocity of 2000nm/s. We obtained maximum unfolding of two repeats.

Polyprotein unfolding of I27

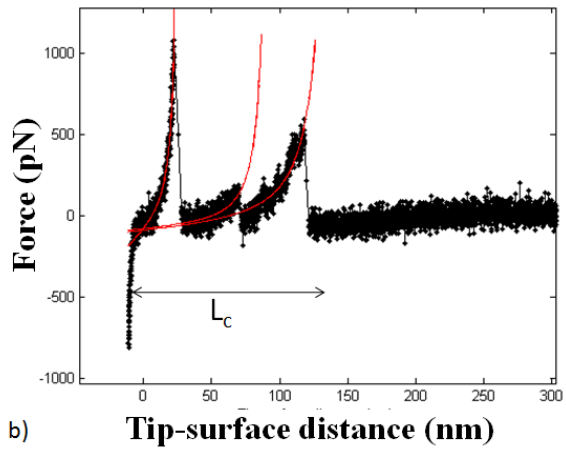


Figure 8a):

Polyprotein unfolding of EC1 cadherin 23

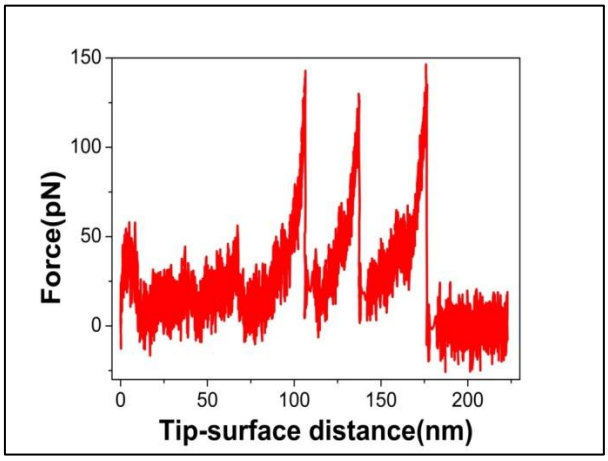


Figure 8b)

a) This is the figure we got after fitting the curve using WLC.It suggests that first unfolding is a PEG stretch and other two are domain unfolding, and the second domain takes higher force to unfold than first.
b) Shows the saw tooth pattern of Cadher23-EC1 unfolding under Force

3.2 Conclusion

We successfully made polyprotein of Cadherin23-EC1 using enzymatic stapling processbut we were unable to get the fingerprint of Cadherin23-EC1 from our force curves. So we made I27 protein as a reference protein. Using our enzymatic stapling process made a polyprotein of 5 repeats of I27. Our unfolding experiments from SMFS did not give any conclusive result as we were able to unfold only two domains.

3.3 Future work

Now we are going to do the sandwich model by keeping the protein of our interest EC1 cadherin 23 and S47P mutant of EC1 cadherin 23 in the middle of the construct and do AFM. We predict that we will be able retrieve detailed fingerprint of these proteins.

3.4Bibliography:

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