ENZYMATIC STAPLING: A NEW METHOD TO MAKE POLYPROTEIN

Yengkhom Sunanda

A dissertation submitted for the partial fulfillment of

BS-MS dual degree in Science

Indian Institute of Science Education and Research Mohali

April 2016

Certificate of Examination

This is to certify that the dissertation titled "Enzymatic Stapling: A new method to make polyprotein" submitted by Ms. Yengkhom Sunanda (Reg. No. -MS11085) for the partial fulfilment 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. Arijit Kumar De Dr. Purnananda Guptasarma Dr. Sabyasachi Rakshit

(Supervisor)

Dated: 22nd April 2016

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Sabyasachi Rakshit at the Indian Institute of Science Education and Research, Mohali.

This 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 acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Yengkhom Sunanda

(Candidate)

Dated: April 22, 2016

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

Dr. Sabyasachi Rakshit
(Supervisor)

Acknowledgement

My heartfelt gratitude goes to Dr. Sabyasachi Rakshit who has given enough guidance throughout the time. I will not be forgiven if I do not thank my lab members, Deb, Jesse, Jagadish, Gayathri, Naimat, Shweta, Ankush, Surbhi who have been supportive enough in carrying out this project. I also thank Muskaan and Dr. Sharvan Sherawat's lab members who have helped in many ways.

Yengkhom Sunanda
April, 2016

List of figures

NOTATION

Abbreviations	Full form
SMFS	Single Molecule Force Spectroscopy
AFM	Atomic Force Microscopy
LASER	Light Amplification by Stimulated Emission of Radiation
EC1	Extracellular Domain 1
DNA	Deoxyribonucleic acid
Cdh23	Cadherin23
PCdh15	Protocadherin15
PCR	Polymerase Chain Reaction
TIRFM	Total Internal Reflection Fluorescence Microscopy
EGTA	Ethylene Glycol Tetra Acetic Acid

Contents

Abstract

Human ear receives varying forces in the form of sound waves creating oscillations in the inner ear fluid. Oscillations in fluid deflect stereocilia located atop of hair-cells. Adjacent stereocilia are connected to each other with tip-links at their tip. Tip-links is formed by a pair of proteins. It is hypothesized that tip-links that serve as gated-spring in the hearing, pull open ion-channels in response to force as sound stimuli. The opening of ion-channel is delicately balanced between the viscoelastic property of the tip-links and wide variations in force experienced by tip-links due to varying intensity of sound. The overall objective of my work is to understand the molecular elasticity of tip-links in the presence of tensile force. We developed a new method of making polyproteins. We use single molecule force spectroscopy with Atomic force microscope to study the force-dependent molecular elasticity of tip-links.

CHAPTER 1

INTRODUCTION

Hearing Mechanism:

Human ear consists of three parts: outer ear, middle ear and inner ear[1]. It receives varying forces in the form of sound waves. The sound stimuli pass through the external auditory canal and transmitted to middle ear's malleus, incus and stapes which then conveyed as pressure to the oval window. The pressure gained by the oval window transmits the sound waves to the cochlear fluid in the inner ear. These sound waves create oscillations inside the ear. The amplitude of the force reaches down to the basilar membrane. Upon the basilar membrane, there is an organ of Corti on top of which comprise the sensory cells called hair cells[2].

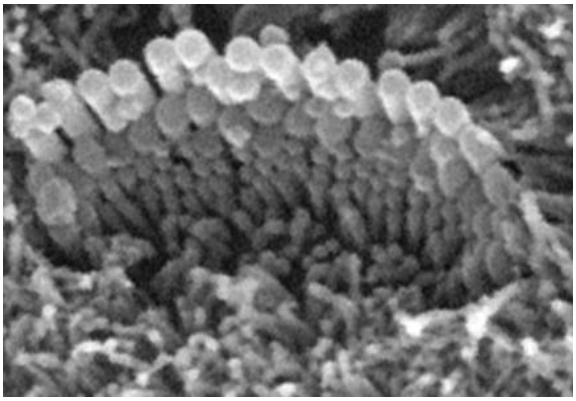


Fig1A. Electron microscope image of stereocilia on a hair cell[3].

The stereocilia protruded from the apical surface of the hair cells are very sensitive to mechanical stimuli[1]. These hair bundles by stereo-cilia are arranged in the increasing order of their heights leading to the formation of bilaterally symmetric structures[4]. On top of each stereo-cilium, ion channels are present through which ion influx occurs and electrical signals are conveyed to the brain via nerve cells[5]. These stereo-cilia are connected to each other by tip-links formation through the interactions of two proteins, Cadherin23 and Protocadherin15[6]. When our ear receives sound stimuli, the stereo-cilia get deflected. The ion channel present on top of stereo-cilia opens and sends signal to the brain. Thus it is believed

that these stereo-cilia act as mechano-receptive organelles which are directly responsible for the mechano-transduction in hearing[7].

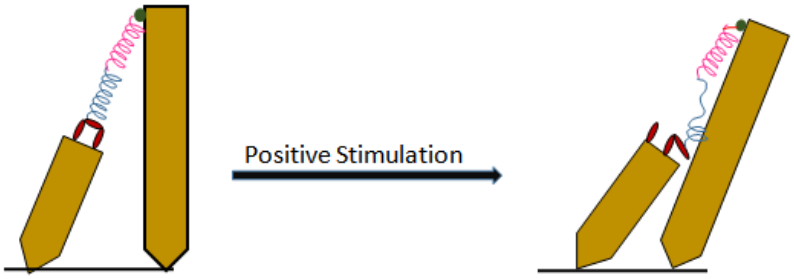


Fig.1B: Tip-links opening the ion-channels due to the sound stimulation

Hypothesis:

Our hypothesis is that these tip-links act as a gated spring in opening the ion channels. It requires a certain threshold force to open the ion channel. Human ear can perceive sounds of frequency ranges from 20 Hz to 20 kHz and of intensity ranging from 5 dB to 120 dB. If the pressure or force exerted by the 5dB sound is able to open the ion channel then what happens to the force exerted beyond the threshold and 120dB. 120dB is expected to create pressure of tip-links of nearly 10^7 orders higher than 5dB. So, the question is, how do the tip-links behave when we receive sound beyond 5dB. Do the tip-links exert the entire force to open the ion channels? Ion-channels are embedded on cell membrane which has an elasticity of 10^2 orders lower than that of the tip-links. If the force is exerted directly on the ion-channels by the tip-links then that can rupture the cell membrane or cause permanent deafness which normally does not happen. We hear sound effortlessly even after listening to rock-band concerts for several hours. In such concerts, the intensity of the sound reaches even beyond 60dB. We hypothesize that the extra forces are dissipated in the unfolding of tip-links which is acting as a gated spring. These stretched proteins regain back to their initial conformation during relaxation. Thus the opening of the ion-channels is balanced by the elastic property of the tip-links through various intensities of sound[8]. The overall objective of this work is to understand the molecular elasticity of the proteins as tip links in presence of tensile force.

For this purpose, we plan to use SMFS with an Atomic Force Microscopy to understand the force dependent unfolding of the tip-link proteins.

Single Molecule Force Microscopy using Atomic Force Spectroscopy:

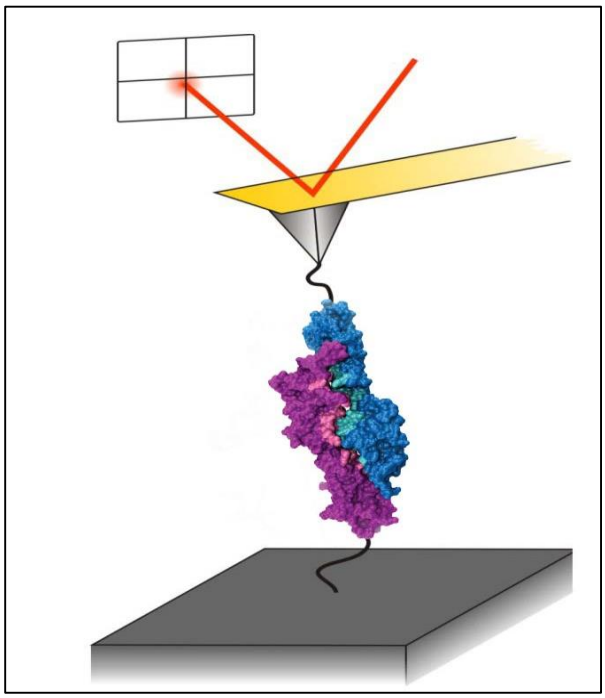


Fig.1C: Picture demonstrating Force Spectroscopy study using AFM

In SMFS using AFM, the AFM cantilever is used as a force sensor. Here the AFM cantilever with or without bait or ligand is moved up and down using piezos. Initially, the cantilever is held up away from the surface and brought down in contact to grab one single molecule at a time. Depending on the binding on-rate of the ligand-receptor interactions, the cantilever is held for varying amount of time. Once the contact is made, the cantilever is pulled away from the surface to exert tensile force on the molecules under probe[9].

The magnitude of force is directly measured by following the bending of the cantilever using a red-LASER bouncing back from cantilever. Execution of tensile force on molecules under probe is usually done in three different ways:

1. Force ramp: Molecules are stretched vertically with constant pulling velocity by the cantilever. This is done for several pulling velocities. In this case, force increases linearly with time[10].
2. Force clamp: Here, molecules are first pulled in a constant velocity and clamped at a constant force until they unfold or unbind. The persistence of the bond or structural integrity is measured directly under varying clamping forces[9-10].
3. Length clamp: The molecule is pulled and stretched until a particular extension/length is reached with forces increasing linearly in time[11].

We obtain the magnitude/strength of the interaction or the entropic and enthalpic energies of folding, lifetime of a bond, thermodynamic and kinetic energy diagram of any ligand receptor interactions/ protein folding, directly or indirectly from such measurements from Force Spectroscopy[12].

SMFS has been extensively used to understand the features of human cardiac titin I27 and I28 modules which unfold in a force dependent manner extending in a step wise procedure[13]. Titin protein is a very gigantic protein found in our muscle. It is considered to be the source of elasticity and flexibility of muscle. When our muscle is stretched due to any heavy work deeds, the thin and thick filaments of the muscle slide past each other. Meanwhile, titin tries to resist it by its resting force and contracts the filaments. This property of titin protein makes itself store potential energy in the muscle. Its mechanical property is well characterized by studying the SMFS.

The use of SMFS is highlighted in the study of ankyrin repeats which is also a huge protein reported to be acting as ion gating spring of mechanoreceptors in *Drosophila bristles*. Thereafter, studies have been done to understand the elasticity of this protein using SMFS showing its spring constant as 4.1pNm^{-1} and unfolding at 240pN[14].

Another example that can set as a possibility towards the use of force spectroscopy is the study of calcium dependent protein Calmodulin. The folding rate is much faster at higher calcium concentration than the unfolding rate. The individual domains functions differently at low concentrations of the calcium.

The idea of making polyprotein is to avoid the non-specific pulling of the single molecule. This will ensure the specific pulling of the molecules under probe. However, using I27 as a marker, one can study the unfolding of one EC1 domain by inserting in between the I27 domains. There, the step wise unfolding can be studied using SMFS.

Tip-link proteins, Cadherin23 and Protocadherin15 are composed of 27 extracellular domains and 11 extracellular domains respectively. The linker region of each domain is bound with three Ca^{2+} ions, ought to be maintaining the rigidity of the proteins[15]. However there is no experimental evidence of these roles of Ca^{2+} ions in these tip-link proteins. In addition to it, Cdh23 has another Ca^{2+} ion binding site near the N-terminal thought to be maintaining the integrity of the protein. Dealing with only Cdh23, I am interested to study the elasticity of the protein with or without Ca^{2+} ions under tensile force. I also intend to extend my study to decipher the importance of Ca^{2+} ions at different binding sites. In order to study these, we design different constructs with or without linker region in the EC1 domain of Cdh23. Since the stepwise unfolding mechanism of EC1 domain is not known yet, it is not possible for us to understand whether it is the molecule under probe or not. To understand the unfolding mechanism, we regard polyprotein as a marker in studying force spectroscopy.

Objectives:

Overall, I have two objectives:

(1) Constructing the polyprotein of EC1 domain of Cdh23 with or without Ca^{2+} linker region and

(2) Deciphering the molecular elasticity of the EC1 domain of Cdh23 with or without Ca^{2+} ions using SMFS.

Approaches:

To achieve the first goal, we have established two different approaches:

1. **Iterative molecular cloning method**: Recombinant DNA techniques are used to synthesize each extracellular domain's constructs. This multistep cloning technique makes use of four restriction enzymes (Nde1, BglII, BamH1 and Xho1) to construct five repeats of Cdh23's extracellular domain 1.

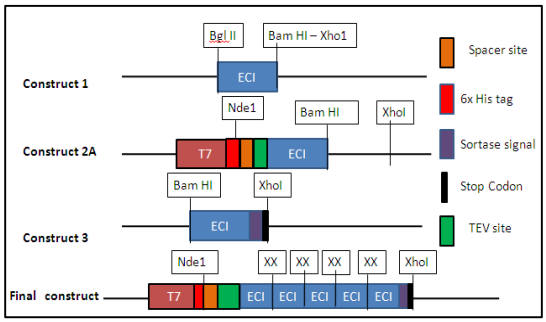


Fig 1D: Block construct of the polyprotein.

T7: T7 promoter

XX: restriction overhangings

2. **Alternative Strategy: Enzymatic Stapling:**

To avoid the heavy time consumption and lengthy process of constructing polyproteins through traditional molecular biology, a relatively cheaper, easier and less time consuming method is designed and adopted to construct the polyprotein – ENZYMATIC STAPLING OF PROTEINS.

Here, an enzyme from Sortase family will catalyze a transpeptidation reaction and ligate proteins sequence specifically by immobilizing[16] them on surfaces.

Chapter 2

METHOD 1: EXPERIMENTAL METHODS, RESULTS and DISCUSSION

Introduction:

DNA Engineering: Recombinant DNA techniques are used to synthesize each extracellular domain's constructs through iterative molecular cloning method. The genes encoding these constructs represented below are amplified using Polymerase Chain Reaction method. The destination vector which is used for cloning is empty pET21 vector. It has multiple cloning sites including all the restriction enzyme sites mentioned here. This multistep cloning technique makes use of four restriction enzymes (Nde1, BglII, BamH1 and Xho1) to construct five repeats of Cdh23's extracellular domain 1 (EC1).

The BamH1 and BglII sites can produce compatible cohesive ends to clone itself over and over again for three times where on the upstream will be filled with construct 2 and downstream by construct 3 to finally construct the 5 repeatative EC1 domains of Cdh23(figure 1D).

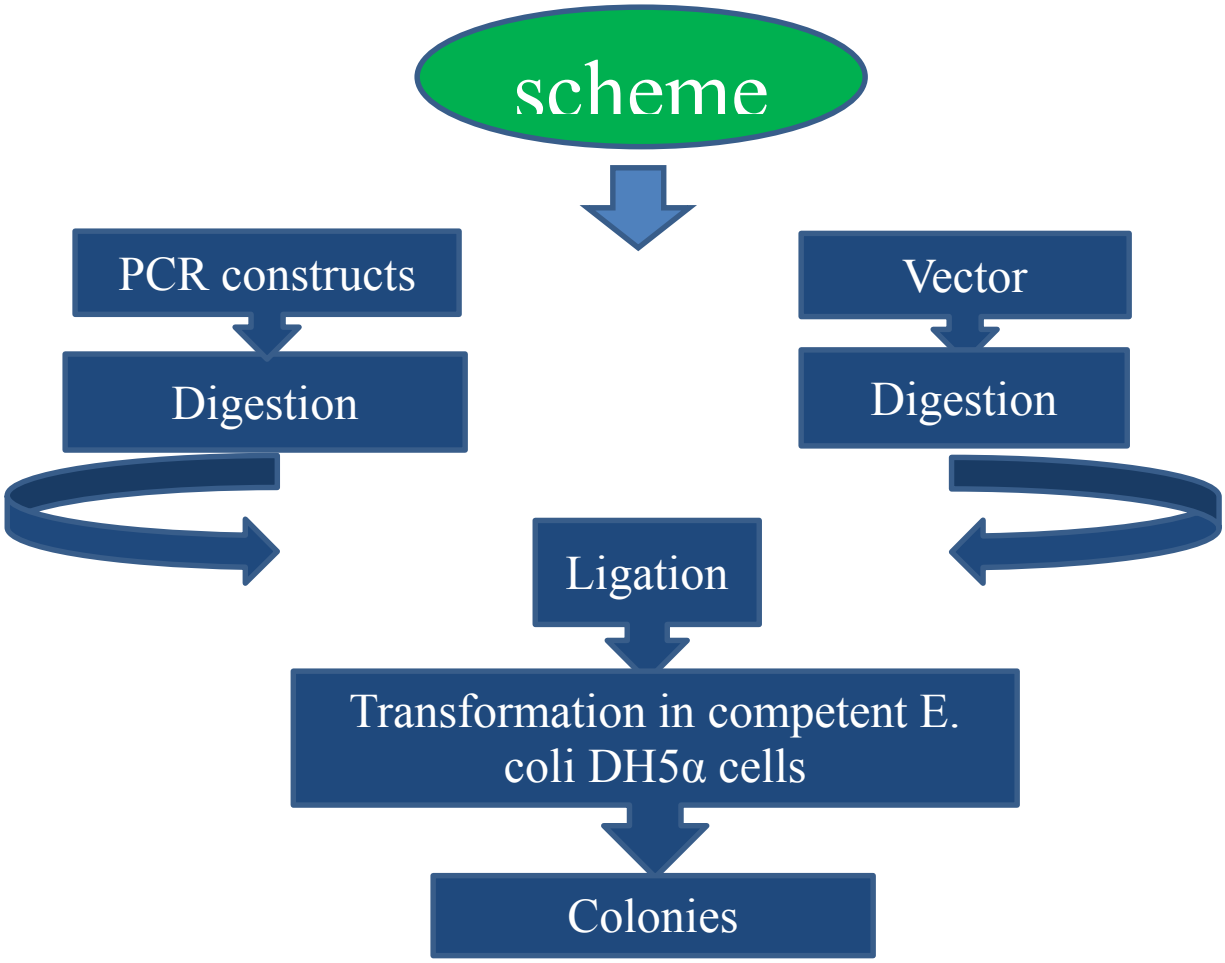


Fig.2A: Schematic diagram of molecular cloning procedure

Material and Methods:

Using EC1 and EC2 containing plasmid as the DNA template, the genes encoding for the three constructs are amplified. These constructs will then be cloned in DH5α strain of *E. coli* competent cells.

Construct 1 Preparation:

1. Insert's PCR amplification:

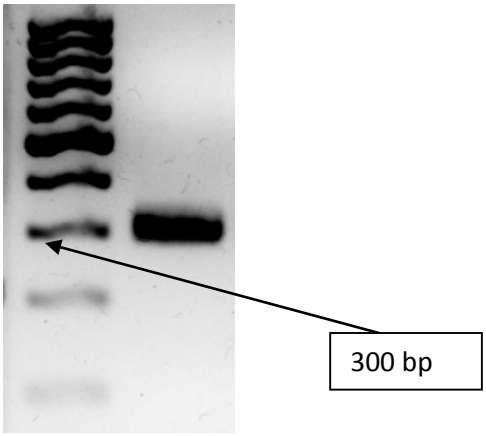


Fig. 2B: Gel picture indicating construct 1 amplification

2. The digestion of the PCR construct and vector are done alongside with restriction enzymes BglII and Xho1 separately and ran in the gel. The gel pictures showing their correct sizes are attached below:

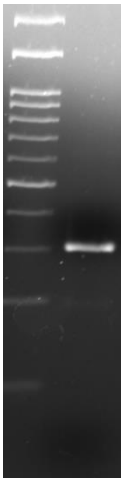


Fig 2C : cut insert

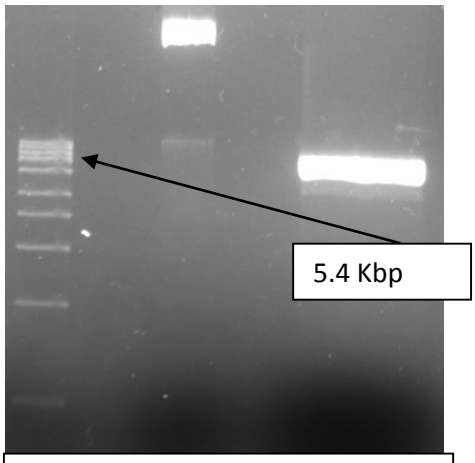


Fig.2D: Cut and uncut vector

The DNAs are extracted and purified from the gel using Invitrogen's Gel Extraction and Purification Kit.

3. Before the ligation mixture is set up, it is very important to ensure if the purified sample that is collected is the desired sample. So a pre-ligation check is set up and ran in the gel.

4. Knowing that purification is successfully done, ligation is set up with vector: insert in 1:4 molar ratio at 16 °C overnight.
5. Transformation is done in DH5α strain of *E. coli* using 10 µL of the ligation mixture in 100 µL of the competent cells.

   The colonies obtained from the transformation are streaked and exposed to 37°C overnight. Following this, plasmid mini-prep is performed using Invitrogen's Mini-Prep Kit and followed by digestion of the plasmid. The result is shown below:

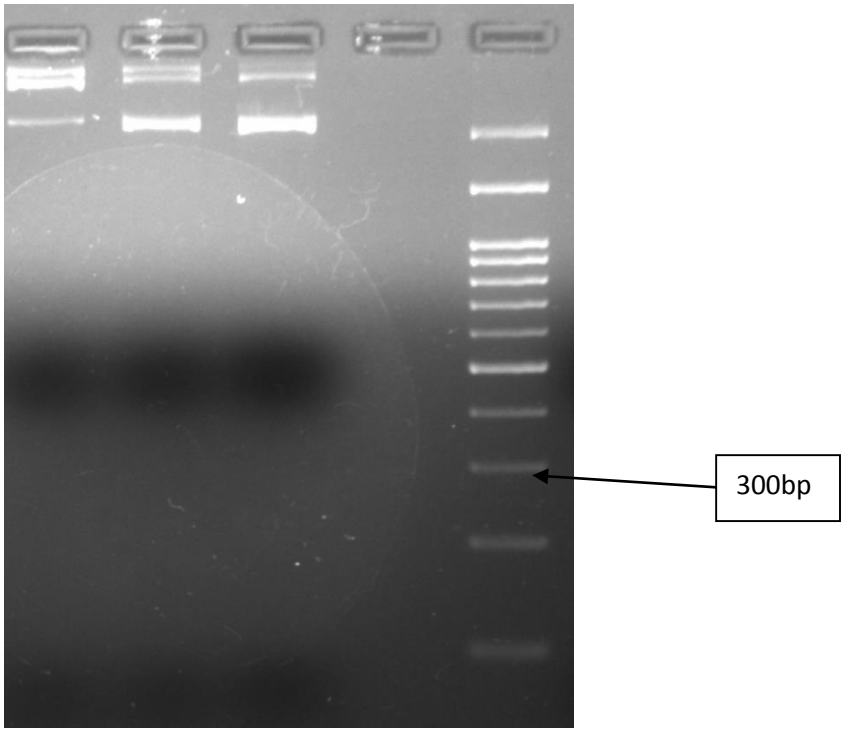


Fig. 2E: Digestion of the cloned plasmid for construct 2.

There is no sign of the presence of the required insert in the plasmid. Therefore, experiment is repeated again but unfortunately no fruitful results came in our hand.

WHAT COULD BE THE PROBLEM??

Construct 2 preparation:

1. Insert amplification:

The amplicon size of the construct 2 insert is 360bp.

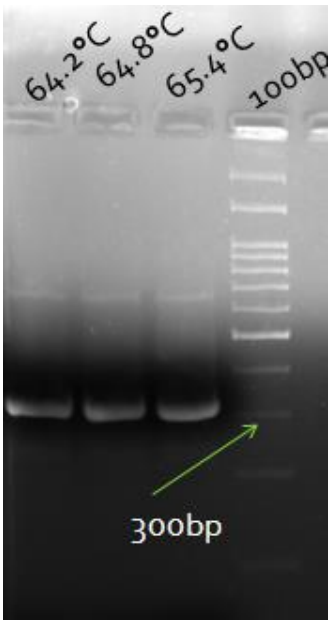


Fig. 2F: Gradient PCR for construct 2

2. The digestion of the vector and insert are done separately with restriction enzymes, Nde1 and Xho1.
3. The pre-ligation check has defined the DNA fragments as candidates to undergo ligation.
4. Similar to that of first construct, ligation of 20 µL is performed overnight at 16°C.
5. Transformation is done consuming 10 µL to 100 µL of the same cells. After the transformation is done, colony PCR is performed as the number of colonies obtained is larger in this case.

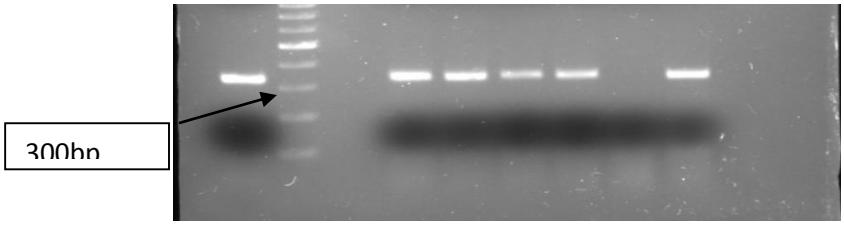


Fig.2G: Colony PCR for construct 2

The plasmid mini-prep is done for four of the colonies that are obtained. After the digestion of these plasmids and running in gel, the result is shown as below:

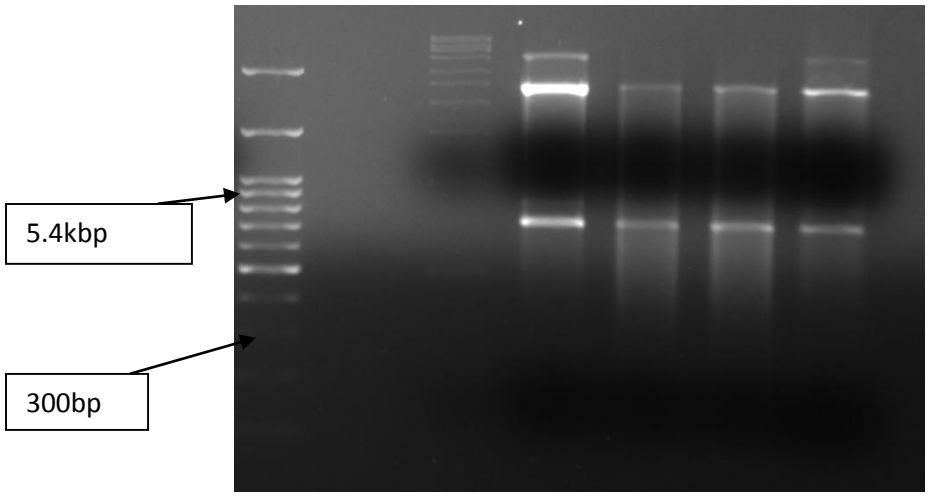


Fig. 2H: Digestion of the cloned plasmid for construct 2

Lane 1 = 100bp ladder
Lane 3 = 1kb ladder
Lane 4-7 = digested plasmids

For the second construct also, it is failing to obtain the desired insert size. The DNA sequencing result gave no sign of the presence of the insert even though the PCR colony gave positive result.

The problem is not understandable so far.

However, I moved on to synthesize the last construct even though the first two construct's syntheses are not concluded so as to figure out if the problem still prevails in the construction of the third construct.

Construct 3 preparation:

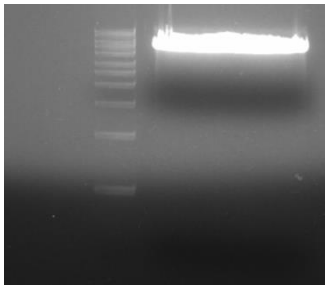
1. Insert amplification:

   The size of the third construct including all the extended sites is 336 bp.

The insert size showing it is nearly

Fig.2I: PCR amplification of the 3rd construct.

2. The digestion of the construct and vector are done separately with restriction enzymes BamH1 and Xho1. The gel picture of the digestion result is presented below:



The cut vector is shown on the left. The digestion of the insert is not run in gel and instead purified directly using Invitrogen's DNA Purification Kit. The pre-ligation check is shown below.

Fig. 2J: Digestion of the vector with BamH1 and Xho1.

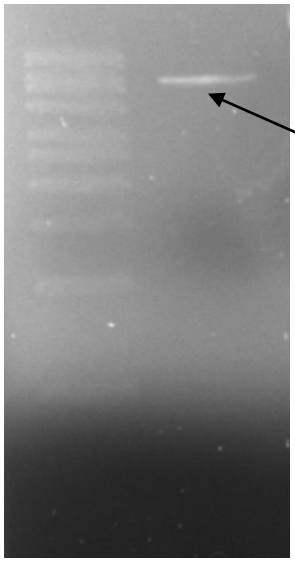
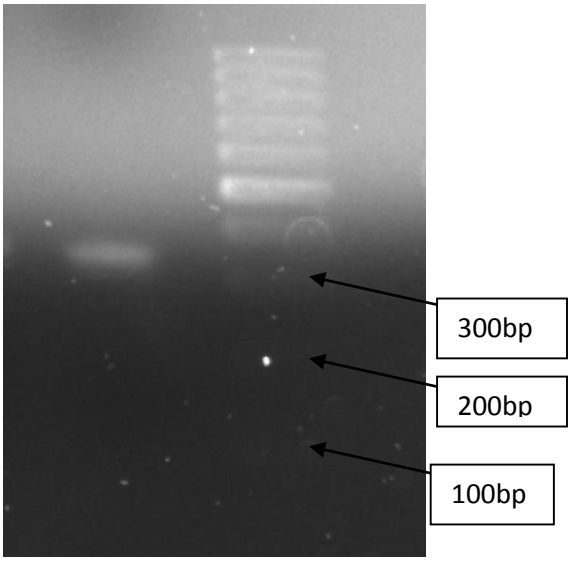


Fig:2K: Picture on the left shows the pre-ligation check of the purified vector to be ligated.

Fig.: 2L Picture below shows the pre-ligation check of the insert to be ligated.



3. Ligation is done at the same temperature for same amount of time followed by transformation. The same protocol is followed in the making of this construct.
4. Colony PCR is done for the colonies obtained.

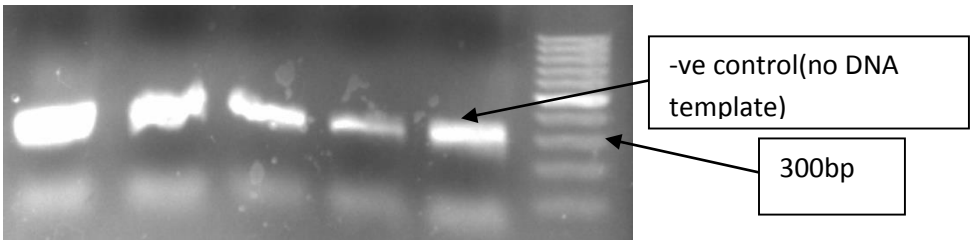


Fig.2M: Colony PCR for construct 3

Since the negative control also gave me specific band, the one which showed thicker specific band is selected for plasmid mini-prep.

Digestion of that plasmid is done and the result is shown below:

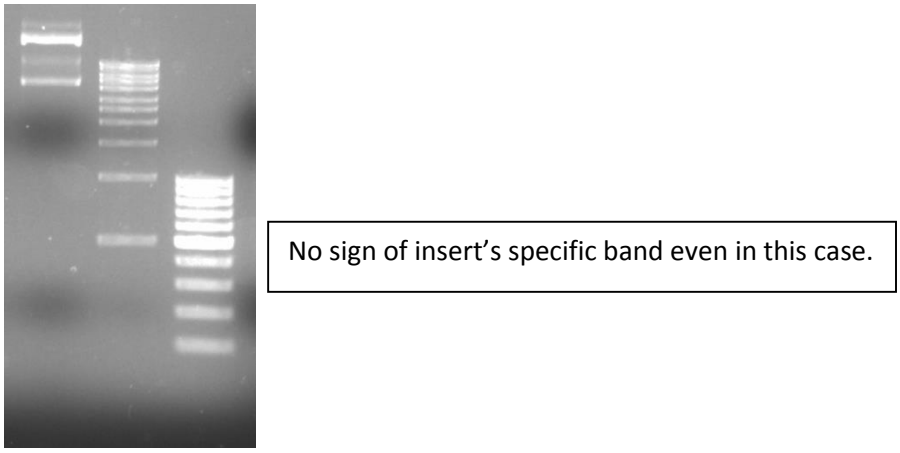


Fig. 2N: Digestion of the cloned plasmid for construct 3

So far, not a single successful clone is able to be made. Efforts have been tried to figure out the problem.

Results and Discussion: The entire work of making polyprotein through this method of recombinant DNA techniques was not entirely a fruitful one. Probable reasons may be because of non functional of restriction endonucleases or the restriction overhangings at the ends are not able to provide sufficient seat for the endonucleases to sit on. Another possibility could be due to the non-functional of empty plasmid due to which it has incredibly failed us in cloning.

CHAPTER 3

METHOD 2: EXPERIMENTAL METHODS AND RESULTS

Introduction:

As an alternative strategy to construct polyprotein, we have tried to develop a new method which is very efficient and effective- ENZYMATIC STAPLING OF PROTEINS.

The Sortase A has the potential to catalyze ligation of proteins in a recognized specific sequence and undergo transpeptidation reaction by immobilizing the protein on surfaces. The recognized sequence is LPETGSS and it is used as a tag on the protein in its C terminal while GGG and 6xHistag in its N terminal.

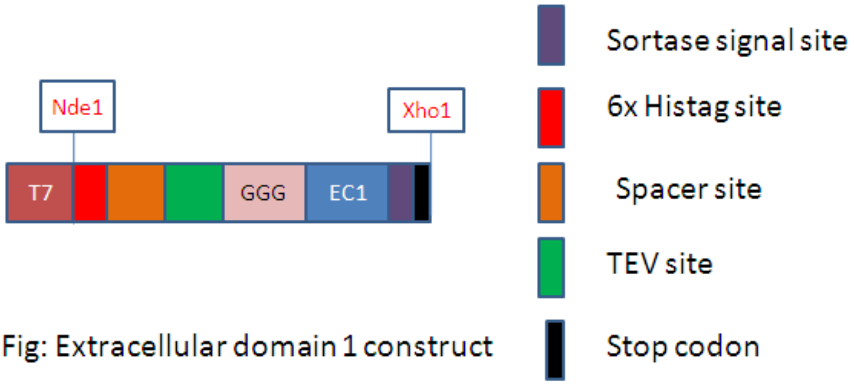


Fig: Extracellular domain 1 construct

Fig. 3A: Construct of Extracellular domain 1

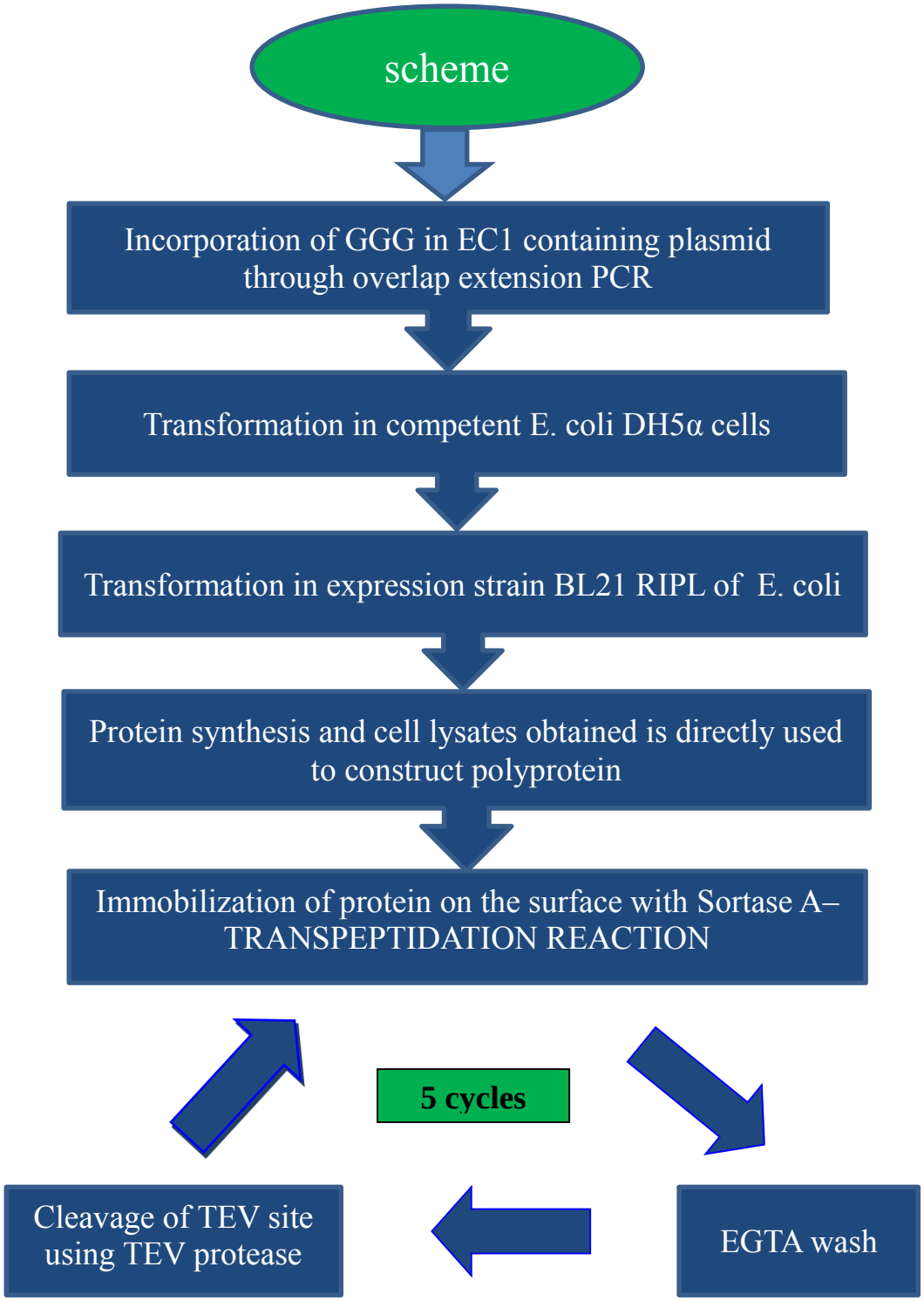


Fig. 3B: Scheme of the reaction

The Cys residue of Sortase A enzyme attacks the amide bond between the threonine and the glycine to form thio-ester bond which is an acyl-enzyme intermediate and is found to be stable at pH 7.4. This intermediate is broken by the nucleophilic attack by the amino group of the polyglycine which subsequently leads to the formation of LPET-GGG bond cleaving the Sortase enzyme.

The polyglycine GGG is required to be exposed for another set of protein to get immobilized on the surface. Hence, the sequences which are on the upstream of the GGG need to be cleaved off. To leave GGG open, TEV protease sits on the TEV site and liberates those sequences. Thus, the formation of polyprotein is enhanced and made easier through this method of stapling Sortase on the protein.

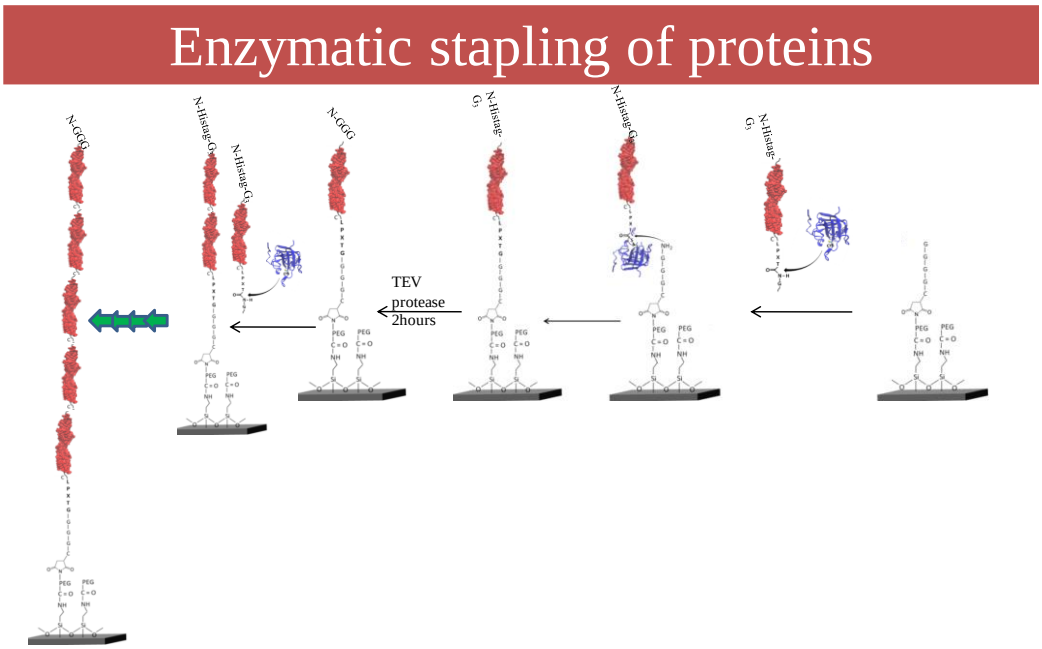


Fig. 3C: Schematic diagram of polyprotein construction using enzymatic stapling of proteins.

The whole process is monitored using TIRF Microscopy using the science of immunohistochemistry in which the antigens and antibodies come towards each other and interact non-covalently through the epitope of the antibody. The antibody will be labeled with a fluorophore called 488Alexa fluorophore to be able to detect when it is shine with fluorescence light.

Material and Methods for the Enzymatic Stapling Method:

The DNA construct of the following EC1 domain was given to me by Jesse and from there the GGG is incorporated using Overlap Extension PCR.

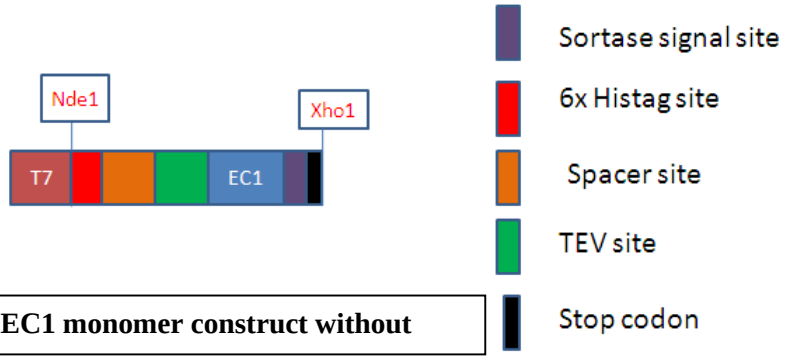


Fig.3D: EC1 monomer construct without

1. The final amplification of EC1 with the incorporation of GGG is done. The size of the construct is 457bp.

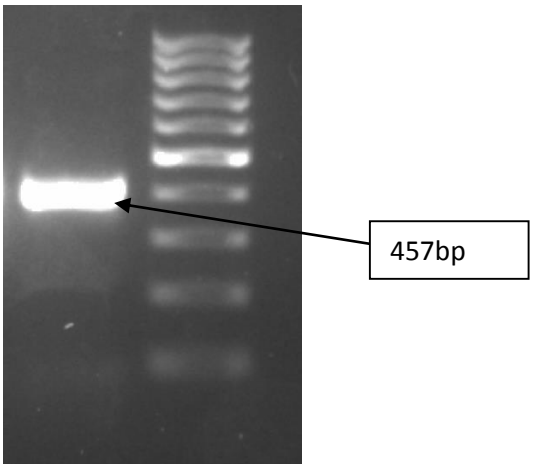


Fig.3E: PCR construct of EC1

2. To insert this DNA fragment inside the plasmid, it needs to be transformed in a cell line. First, digestion of the plasmid pET21 containing EC1 is considered as the destination vector.

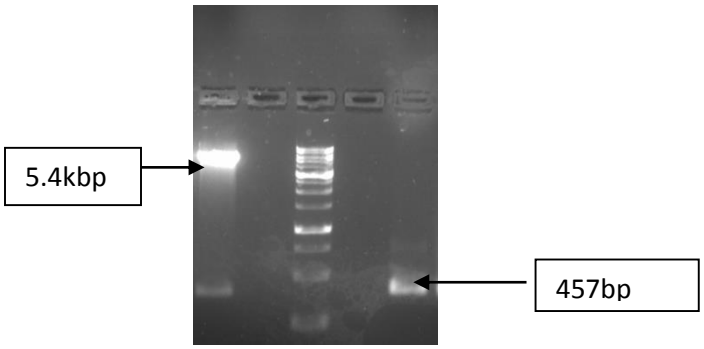


Fig.3F: Digestion of the vector and insert for the EC1 monomer with GGG

3. After the transformation, with the colonies obtained plasmid mini-prep is done and double digestion is done for them.

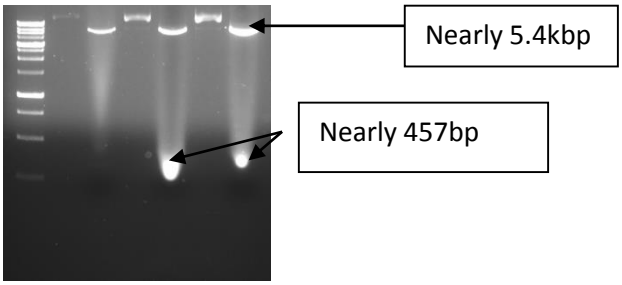
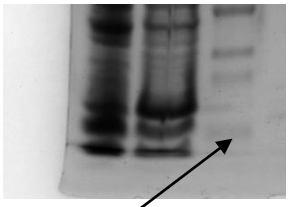


Fig. 3G: Double digestion of the cloned plasmid showing positive result.

Protein Expression:



Bacterial culture is grown at 37°C until the OD_{600} is reached. The protein-prep is done with the IPTG induction of 100mM at 20°C overnight after the OD_{600} reaches 0.5. the culture is pelleted down and resuspended using resuspension buffer. The cell lysates is run on the gel and used for the polyprotein synthesis.

Nearly 17kD

Fig.3H: SDS PAGE of the Cdh23 EC1 with GGG

Construction of polyproteins:

Pegylation of 1% PEG maleimide in SVA PEG is done for four hours on the cover slip of silicon nitride. GGGGC nucleophile is incubated on the surface for 7 hours .

1. Surface is incubated with Cadherin23 EC1 monomer protein with Sortase A(250nM) for an hour.
2. After the incubation is done, the surface is washed off to remove all those proteins which have failed to get immobilized.
3. TEV protease is incubated for 2 hours to cut the TEV site so that GGG from the protein is exposed.
4. After the incubation is done, the surface is washed vigorously.
5. Next set of Cdh23 protein and Sortase A are incubated for one hour and the experiment is repeated until the formation of five domains.

This whole experiment is monitored using immunohistochemistry in TIRF Microscopy. Primary antibody(anti-polyhis from rabbit,1:1000 dilution) is incubated on the surface for an hour after step1. Secondary antibody (antibody from goat for the above antibody, 1:1000 dilution) which is already labeled with 488Alexa Flourophore is then incubated for an hour to covalently attach on the former antibody and did imaging.

Vigorous washing is done and then since our protein is Ca^{2+} dependent and has the tendency to form homodimer, EGTA washing/incubation is done for half an hour and did imaging,

TEV protease is incubated to cut the TEV site exposing GGG and did imaging.

The whole experiment is repeated form step 1 to form another layer on the surface and goes on until the fifth layer is formed.

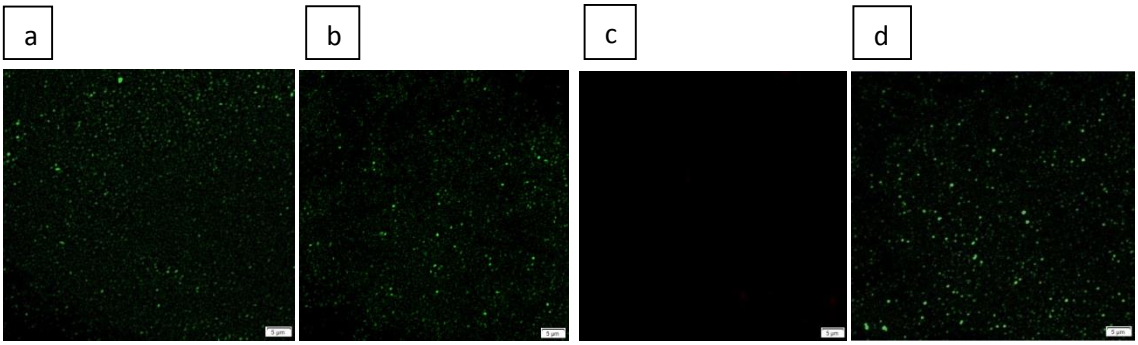


Fig. 3I: TIRF Images.

a. antibody labeled Cdh23 with 6xhistag at N terminus.

b. after EGTA wash.

c. after TEV cut.

d. antibody labeled Cdh23 with 6xhistag at N terminus of next layer.

The observation of signals in Fig.3F.a and b means that our protein is immobilized on the surface and the EGTA wash has removed those non-immobilized Cdh23. The absence of signal in Fig.3F.c shows that the TEV cut is properly done and GGG is exposed. The presence of signal in Fig.3F.d means that the second layer polyprotein of Cdh23 is formed successfully.

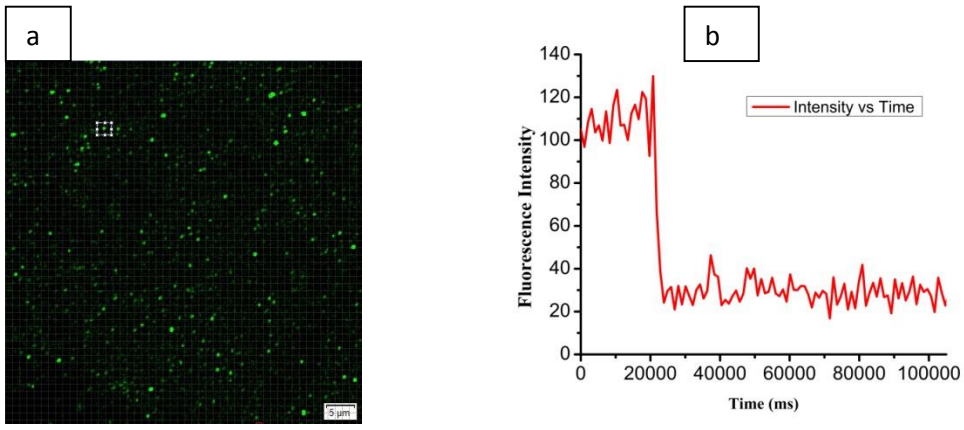


Fig.3J: Photobleaching image and its intensity Vs lifetime profile.

a. **The square bracket spot on top left is a photobleaching of single molecule Cdh23 first layer.**
b. **There is a sharp decrease in the intensity Vs lifetime profile showing that it's a single molecule photobleaching.**

Thus, through this method we could monitor the formation of polyprotein.

To confirm the formation of polyprotein and study the unfolding mechanism of the five EC1 repeats, force ramp spectroscopy is done. Various loading rates are given to unfold the protein from 400pN/s to 4000pN/s.

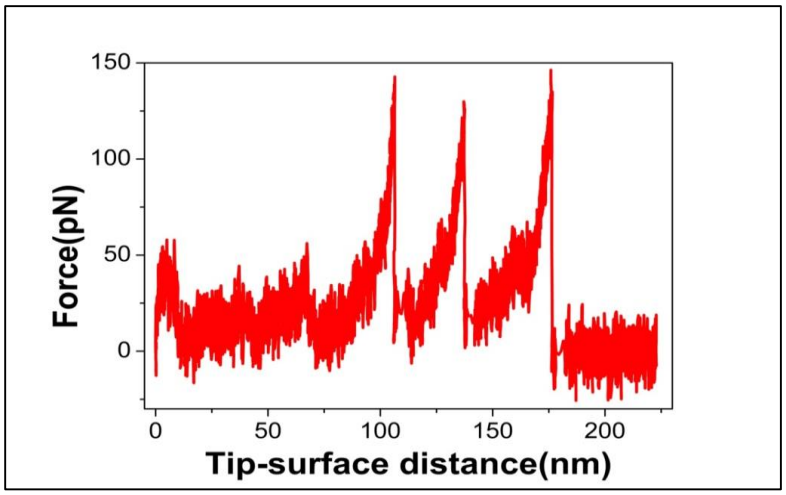


Fig. 3K: Force curve: saw tooth pattern of force curves indicating force vs extension profile showing unfolding of three domains.

Since the cantilever is not modified with anything, it has equal probability of picking up and stretching any non-specific particles as well. So, the cantilever must have picked up from the third domain and stretched it and hence, we could see three domains getting unfolded.

Overall Conclusion:

The traditional molecular cloning method did not work here because of the non-functional of the empty pET21 plasmid but otherwise is efficient and a successful method of making polyprotein. However, it is a tedious job when it comes to the use of many restriction enzymes and constructing more number of domains. To avoid this heavy time consumption, we have designed a new method of constructing polyprotein using Sortase A enzyme in the enzymatic stapling of proteins. This new method has advantages over the traditional method of constructing polyprotein which are cheaper, faster and easier. This enzyme has effectively ligated proteins in a very specific sequence.

The force spectroscopy data has revealed the saw-tooth pattern showing the multiple unfolding of domains. However, in order to understand the mechanical resistance of cdh23 better, we need marker domains like I27 to selectively identify Cdh23 stretching.

Bibliography

1. Slepecky, N. B. Structure of the Mammalian Cochlea. In *The Cochlea*, Dallos, P.; Popper, A. N.; Fay, R. R., Eds. Springer New York: New York, NY, 1996; pp 44-129.
2. LeMasurier, M.; Gillespie, P. G. Hair-cell mechanotransduction and cochlear amplification. *Neuron* **2005,** *48* (3), 403-15.
3. Stauffer, E. A.; Holt, J. R. Sensory transduction and adaptation in inner and outer hair cells of the mouse auditory system. (0022-3077 (Print)).
4. Goodyear, R. J.; Marcotti, W.; Kros, C. J.; Richardson, G. P. Development and properties of stereociliary link types in hair cells of the mouse cochlea. *The Journal of comparative neurology* **2005,** *485* (1), 75-85.
5. Gillespie, P. G.; Walker, R. G. Molecular basis of mechanosensory transduction. *Nature* **2001,** *413* (6852), 194-202.
6. Kazmierczak, P.; Sakaguchi, H.; Tokita, J.; Wilson-Kubalek, E. M.; Milligan, R. A.; Muller, U.; Kachar, B. Cadherin 23 and protocadherin 15 interact to form tip-link filaments in sensory hair cells. *Nature* **2007,** *449* (7158), 87-91.
7. Hudspeth, A. J.; Jacobs, R. Stereocilia mediate transduction in vertebrate hair cells (auditory system/cilium/vestibular system). *Proceedings of the National Academy of Sciences of the United States of America* **1979,** *76* (3), 1506-9.
8. Meyer, J.; Furness Dn Fau - Zenner, H. P.; Zenner Hp Fau - Hackney, C. M.; Hackney Cm Fau - Gummer, A. W.; Gummer, A. W. Evidence for opening of hair-cell transducer channels after tip-link loss. (0270-6474 (Print)).
9. Giannotti, M. I.; Vancso, G. J. Interrogation of single synthetic polymer chains and polysaccharides by AFM-based force spectroscopy. (1439-4235 (Print)).
10. Oberhauser, A. F.; Hansma, P. K.; Carrion-Vazquez, M.; Fernandez, J. M. Stepwise unfolding of titin under force-clamp atomic force microscopy. *Proceedings of the National Academy of Sciences of the United States of America* **2001,** *98* (2), 468-72.
11. Carrion-Vazquez, M.; Oberhauser Af Fau - Fisher, T. E.; Fisher Te Fau - Marszalek, P. E.; Marszalek Pe Fau - Li, H.; Li H Fau - Fernandez, J. M.; Fernandez, J. M. Mechanical design of proteins studied by single-molecule force spectroscopy and protein engineering. (0079-6107 (Print)).

12. Moy, V. T.; Florin El Fau - Gaub, H. E.; Gaub, H. E. Intermolecular forces and energies between ligands and receptors. (0036-8075 (Print)).
13. Oberhauser, A. F.; Hansma Pk Fau - Carrion-Vazquez, M.; Carrion-Vazquez M Fau - Fernandez, J. M.; Fernandez, J. M. Stepwise unfolding of titin under force-clamp atomic force microscopy. (0027-8424 (Print)).
14. Lee, G.; Abdi K Fau - Jiang, Y.; Jiang Y Fau - Michaely, P.; Michaely P Fau - Bennett, V.; Bennett V Fau - Marszalek, P. E.; Marszalek, P. E. Nanospring behaviour of ankyrin repeats. (1476-4687 (Electronic)).
15. Sotomayor, M.; Weihofen Wa Fau - Gaudet, R.; Gaudet R Fau - Corey, D. P.; Corey, D. P. Structural determinants of cadherin-23 function in hearing and deafness. (1097-4199 (Electronic)).
16. Parthasarathy, R.; Subramanian S Fau - Boder, E. T.; Boder, E. T. Sortase A as a novel molecular "stapler" for sequence-specific protein conjugation. (1043-1802 (Print)).