Biochemical Analysis of Chromatin Remodelling Activity Of Type-ISP Restriction Modification Enzyme LlabIII

A Project Submitted to Indian Institute of Science Education and Research (IISER) Pune Towards Fulfillment of Five Years Integrated BS-MS Degree Programme (March 2017)

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Certificate

This is to certify that this dissertation entitled **"Biochemical analysis of chromatin remodeling activity of Type-ISP restriction modification enzymes LlaBIII"** towards the partial fulfilment of the BS-MS Dual Degree Programme at the Indian Institute of Science Education and Research, Pune represents original research carried out by Mr. Nibrasul Haque K.M at Indian Institute of Science Education and Research (IISER Pune) under the supervision of Dr. Saikrishnan Kayarat, Associate Professor, Department of Biology, Indian Institute of Science education and Research (IISER PUNE) during the academic year 2016-2017.

Date: 20-03-2017

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Declaration

I hereby declare that the matter embodied in the report entitled **“‘Biochemical analysis of chromatin remodeling activity of Type-ISP restriction modification enzymes LlaBIII”** are the results of the investigations carried out by me at the Department of Biology, Indian Institute of Science education and Research (IISER PUNE) under the supervision of of Dr. Saikrishnan Kayarat, Associate Professor, Indian Institute of Science education and Research (IISER PUNE) and the same has not been submitted elsewhere for any other degree.

Date:20-3-2017 Mr. Nibrasul Haque

Acknowledgements

I would like to thank my supervisor Dr. Saikrishnan Kayarat (Associate Professor, IISER Pune) for giving me the great opportunity to work on this exciting project. I would also like to thank Dr. Geetha Narlikar (Professor, Biochemistry and Biophysics, University of California, San Francisco) for providing the plasmid constructs for expressing the recombinant histones for my experiments. I would like to thank IISER Pune for providing a wonderful atmosphere which is essential to become a good researcher. I would like to thank Dr. Dileep vasudevan (scientist -C, Department of Biotechnology, Indian Institute of Life Science, Bhubaneshwar) for help in some of the experiments. I would next like to thank Mr. Mahesh k chand (PhD student, IISER Pune) for assistance and training to handle the equipment's used in the project. I would also like to thank Divyang Damor for his sincere help and support in initiating this project.

I would like to thank Dr. Gayathri Pananghat (Assistant Professor, IISER Pune) and for being a great source of academic support. I would like to thank Ishtiyaq khan (PhD student), Jyoti Baranwal (PhD student), and all other lab members from both SK (Saikrishnan) and G3(Gayathri Pananghat) lab for their sincere help and support. I would also like to thank my family members and friends for their constant support and advice which helped me to move forward during the hard times.

Abstract

Type ISP restriction-modification (RM) enzymes are bacterial defense systems that nucleolytically cleave foreign DNA entering a bacterial cell (restriction) and protect the host DNA from being restricted by methylating the host DNA (modification). The nucleolytic activity of the enzyme requires hydrolysis of ATP. Cleavage happens when two Type ISP enzymes loaded on their specific target sequence converge subsequent to ATP-dependent translocation (one-dimensional motion) along the DNA (Chand et al., 2015). Interestingly, the ATPase motor that drives DNA translocation is conserved in other enzymes, including chromatin remodelers, such as SWI/SNF and Ino80 (Hopfner et al., 2012; Narlikar et al., 2013), DNA repair enzyme Rad54 (Thoma et al., 2005) etc. The domains(Figure1) of Type-ISP RM Enzymes are TRG (Target recognition domain), MTase domain (for methylating the foreign DNA), SF2 helicase like ATPase domain (for hydrolysis of ATP) and Nuclease domain (for nuclease activity). Chromatin remodelers help in making changes in interaction between DNA and histone octamer. H2A, H2B, H3, H4 dimers are the subunits of the octamers. The aim of the project was to find if Type ISP enzyme can remodel chromatin. To address this question, nucleosome was assembled as roadblocks on path of the Type ISP enzyme. Remodeling of nucleosome was examined using biochemical assays. The assays helped us in arriving at the conclusion that the TypeISP RM enzyme LlaBIII could remove the roadblock (histone octamer bound to a DNA substrate).

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Introduction

1.1: Type ISP Restriction modification enzymes:

Restriction modification systems are bacterial defense systems against foreign DNA that attacks the bacterial cell. These systems are composed of an endonuclease carrying out the nucleolytic cleavage (restriction) of foreign DNA and methylase, which methylate the host DNA (modification) thus protecting it from digestion (9). For active RM enzymes requires Mg2+ for restriction and S-adenosylmethionine (AdoMet) for modification. Mainly RM system can be divided into three classes Type I, Type II and Type III on the basis of enzyme structure, recognition sequence, location of DNA cleavage relative to recognition sequence and cofactor requirements. Type II RM enzymes exist as separate restriction and modification enzymes that act independently from each other, but which recognize the same the single specific 4-8 bp DNA sequence (10).

Type ISP RM enzymes are bacterial defense mechanism towards foreign DNA that enters the bacterial system. They have the property of protecting the host DNA by methylating it and nucleolytically cleaving the foreign DNA with the help ATP motor as a translocation machinery (1). Both the restriction and modification component are present in the same enzyme. Type ISP restriction enzyme have mainly 4 domains - nuclease domain for nucleolytic activity; SF2 helicase like ATPase domain which has two subdomains, i.e. the N-core and C-core; MTase domain carrying out methylation; TRD as the target recognition domain and an additional coupler domain function as a coupler that joins the restriction and methylation units as show in the figure 1 below. These enzymes are a model for understanding modular, multifunctional protein machines, particularly in formulating concepts of protein-DNA recognition, DNA methylation and base flipping, nuclease activity double-stranded (ds)DNA translocation by superfamily 2 (SF2) helicases and long-range communication by enzymes (1).

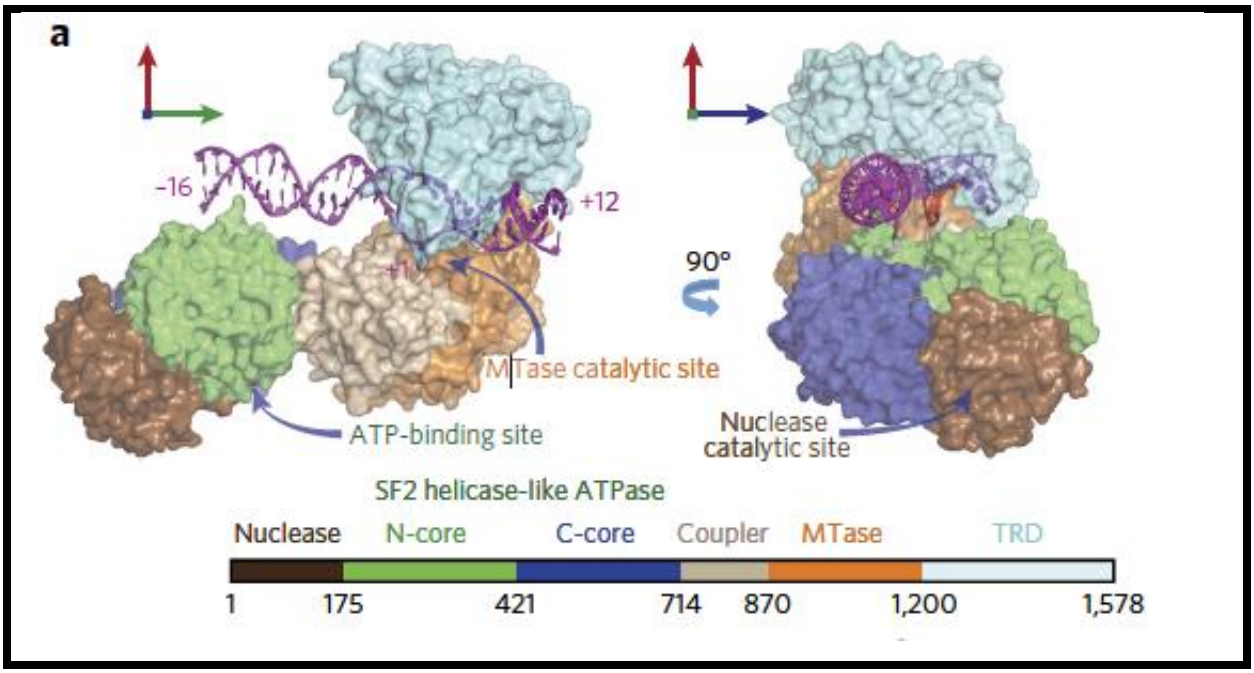


Figure 1: Architecture of Type ISP RM Enzyme bound to DNA. (Adapted from Chand et. al, 2015)

1.2: Chromatin, chromatin remodeling, and chromatin remodelers

Chromatin is the compact structure of DNA wrapped with the help of a histone octameric protein, which is made of 4 protein subunits called H2A, H2B, H3 and H4. The size of human histone octamer, is 108 kDa. The molecular weight of each octameric subunits are H2A - 13 KDa, H2B - 14 KDa, H3 - 15 KDa and H4 - 11 KDa. Basic unit of a chromatin is called a mononucleosome, which has a 147 bp DNA wrapped around one histone octameric unit. Nucleosomal arrays form the chromatin.

The wrapping of genomic DNA as chromatin regulates the expression of the gene. The eukaryotic cell has chromatin remodelers to alter the structure of the chromatin by modulating the assembly of the nucleosome on DNA (12). Chromatin remodelers are of two types. They are ATP dependent and ATP independent chromatin remodelers. ATP independent chromatin remodelers are histone modifying complexes that do acetylation, methylation, phosphorylation and ubiquitination. ATP dependent chromatin remodelers have conserved ATPase domain, they move, eject or modify the histone octamer, such that the site become accessible to other proteins for their binding activity (3). Some

common ATP dependent chromatin remodelers are Rad54, SWI-SNF, ISWI, CHD, INO80 etc. Most of them have many subdomains to recognize whether, where and when to do remodeling in the chromatin.

1.3: Structural similarity of ATPase motor domain of LlaBIII and chromatin remodeler Rad54

Type ISP RM enzyme LlaBIII have unidirectional activity (5'->3'). They go and bind to their target recognition site on the unmethylated foreign DNA that attacks them. Two LlaBIII enzymes bound to target sites arranged in head-to-head orientation translocate along DNA in ATP-dependent manner and collide resulting in cleavage of the foreign DNA (1). Methylation process helps them from self-restriction.

Swi2/SNF2 are involved in various cellular process like DNA repair, cell signaling and regulation of RNA polymerases. To achieve this function, they remodel chromatin which is unwrapping of DNA from nucleosome complex. This chromatin remodeling is achieved by translocation on dsDNA. Recent study by Hopfner et al.,2012 on ISWI says that these enzymes performs screw motion on DNA upon ATP hydrolysis because of which rotational torque is produced which brakes protein DNA interface leading to remodeling of the chromatin.

LlaBIII and Swi2/SNF2 have different cellular role, still they contain a common SF2 helicase core. These contain two conserved RecA like domains. Interface of this RecA like domains contains motifs required for ATP hydrolysis. Figure 2 shows the structural similarity of the SF2 helicase from LlaBIII, Swi2/Snf2 from *Sulfolobus solfataricus* and zebrafish Rad54.The structural studies on these systems shows that they contain conserved catalytic core for ATP hydrolysis and translocation. Here we are interested in knowing that irrespective of the structural similarity do they possess functional similarity too. Consequently, the aim of the project is to study of LlaBIII, a prokaryotic RM enzyme, can displace mononucleosome.

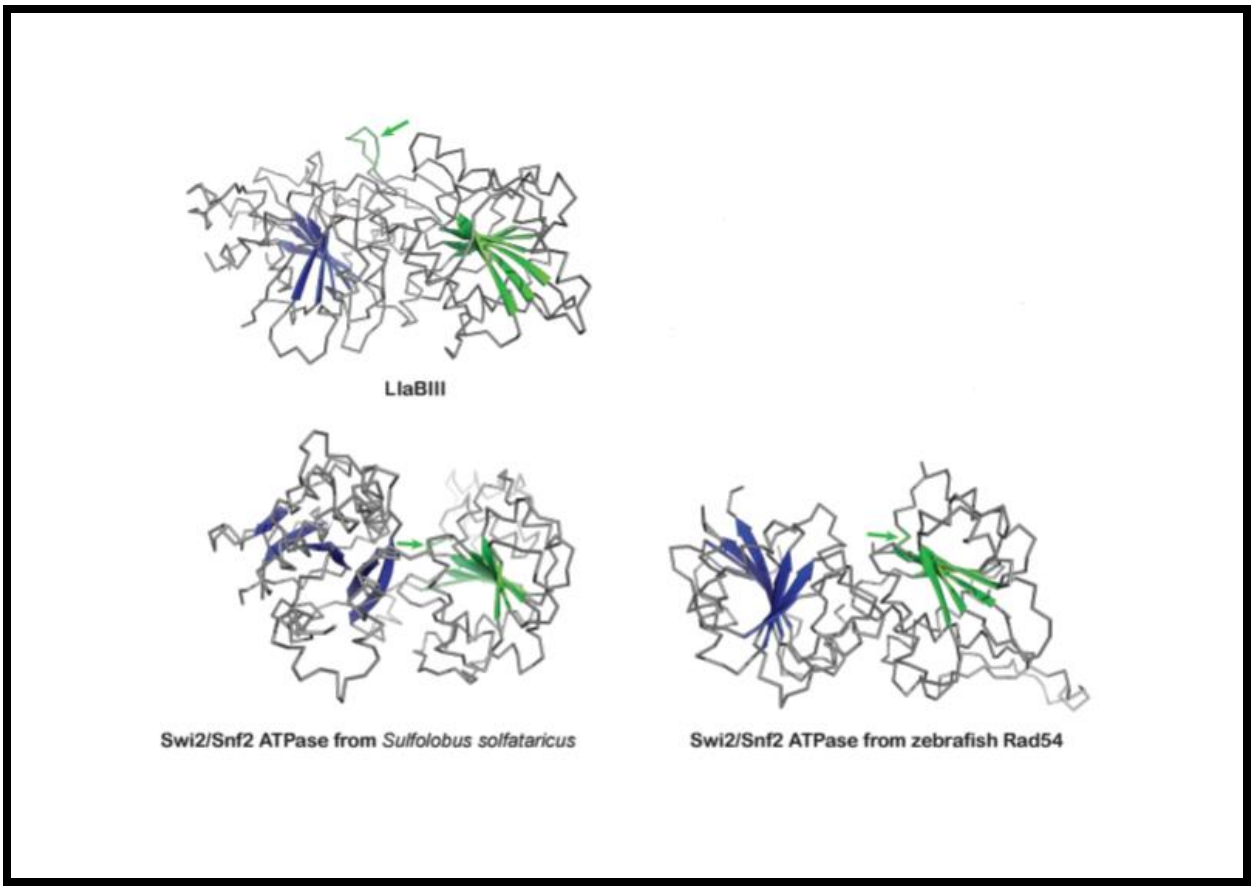


Figure 2: Structural similarity of LlaBIII ATPase domain with Swi/Snf ATPase from *Sulfolobus solfataricus* and Swi/Snf ATPase from Rad54.Diagram highlighting the relative interdomain orientation of the N-core (green) and the C-core (blue) domains of LlaBIII Swi/Snf ATPase from *Sulfolobus solfataricus* and Swi/Snf ATPase from Rad54. (adapted from Chand et al 2015)

Materials and Methods

2.1: Purification of histone octamers H2A, H2B, H3 and H4

H2A, H2B, H3 and H4 are the four basic subunits of histone octamer. H2A, H2B, H3, H4 in PET3 vector were transformed into DH5alpha cells for plasmid preparation and BL21/DE3 cells for expression check. Expression check was done before adding urea and after adding urea. After adding 6M urea (denaturing condition) most of the protein were in soluble fraction. Purification was done by RHP (Rapid Histone Purification) protocol. Both anion exchange (to remove negatively charged impurities) using Q HP Column (5 ml) and cation exchange using SP HP Column (5 ml) was done. Before fraction collection anion column/Q HP was removed and proteins eluted out in 400mM-800mM NaCl concentration.H2A and H3 were purified by my colleague Divyang Damor and H2B, H4 by me. Purified proteins were dialyzed against milli-Q. Proteins were concentrated using lyophilization.

2.2: Octamer assembly and purification from impurities like tetramer, dimer and monomers

For octamer assembly proteins were dissolved together in unfolding buffer (7 M guanidine hydrochloride, 20 mM Tris-Cl pH 7.5, 10 mM DTT) and dialyzed against Refolding buffer (10 mM Tris-Cl pH 7.5, 2 M NaCl, 1 mM EDTA, 5 mM beta-mercaptoethanol) in a dialysis bag with a molecular weight cutoff of 3000 Da. Size exclusion chromatography (superdex 200) was done to get rid of the impurities like histone dimers and tetramers. In a 24 ml column, the histone octamer used to elute at 11-12 ml and in 120 ml column they elute around 60 ml. The pattern of the UV plot that we were expecting is given below and we were using a 24 ml superdex 200 column and expecting that the octamer would elute out around 12 ml and having other protein plots as marker. The size of histone octamer is 108 kDa. The void fractions that elute out are higher order aggregates, then comes the octamers, the shoulder peek shows the tetrameric contaminations and finally the monomeric and small sized contaminants will be eluted. In order to maintain the higher yield of octamer and less contaminants a proper stoichiometry of different subunits have

to be used. If not, the ratio of the octamers with that of the other forms would change. Octamers (figure 2) were pooled and concentrated, flash frozen and stored at -80 °C. The actual concentration of octamer was measured by multiplying the molar extinction coefficient with the value obtained from the spectrophotometer nanodrop. The purified histone octamers can be stored at -30 after adding 50% glycerol to avoid effects of temperature fluctuations. The purified histone octamers were analyzed on a 15 or 18% gel.

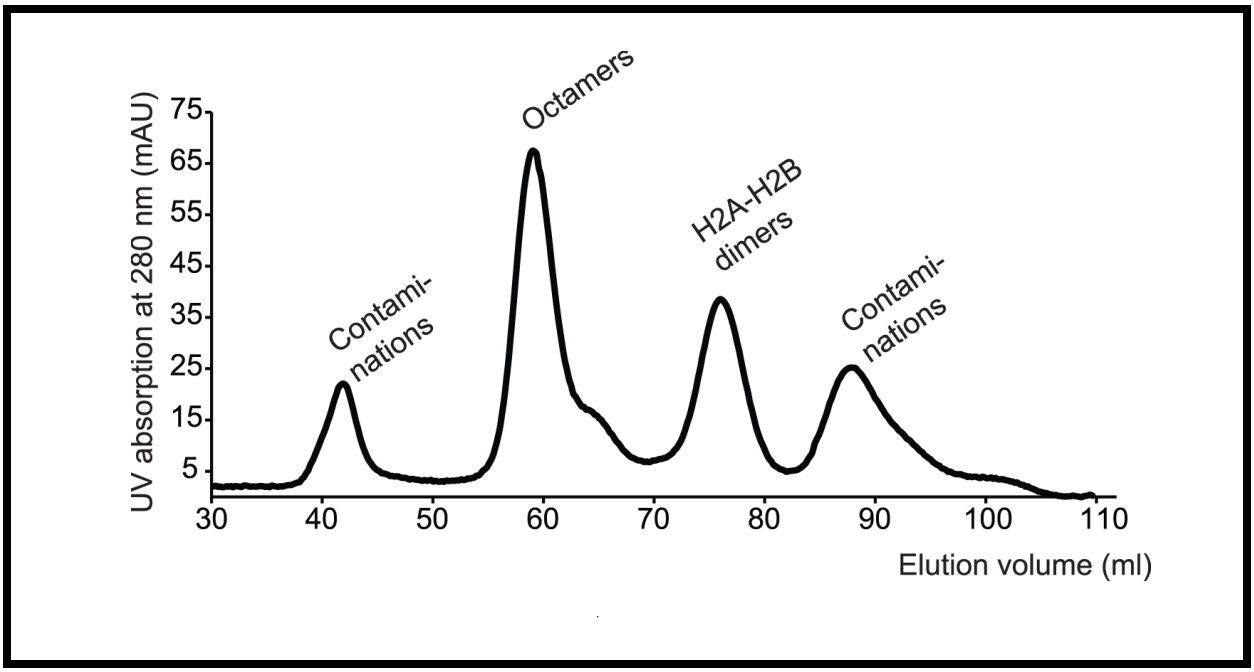


Figure 3: Histone octamer purification UV plot for superdex 200(RHP) (Henrike et al., Plos one,2014)

2.3: DNA substrate that we designed for our biochemical studies

The DNA substrate that we designed for our biochemical assay have recognition site for Type ISP enzyme LlaBIII (Figure 4). In case of LlaBIII, the enzyme requires atleast 23 bp upstream of the target site for their proper seating in DNA Substrate and initiating translocation (Chand et al., 2015). Hence, the substrate that we designed was placed 30bp downstream of one of the ends (Figure 4). The separation between the target site and 601 sequence was 48 bp (Figure 4) to avoid any steric hindrance to the binding of

LlaBIII to its target site. The DNA substrate also had an HhaI site in the middle of 601 sequence. In the mononucleosome complex, the HhaI site will be hidden by the histone octamer.

As a consequence of the translocase activity of LlaBIII the mononucleosome is displaced then the HhaI site will be exposed. In presence of HhaI, the 238 bp long DNA will be cleaved resulting in 164bp and a 74bp fragments.

We already had 601 sequence inserted in a PET3 vector, so we ordered the forward primer and reverse primers required for the PCR amplification of both 147bp 601 sequence and 238bp DNA that has to be used for our assays.

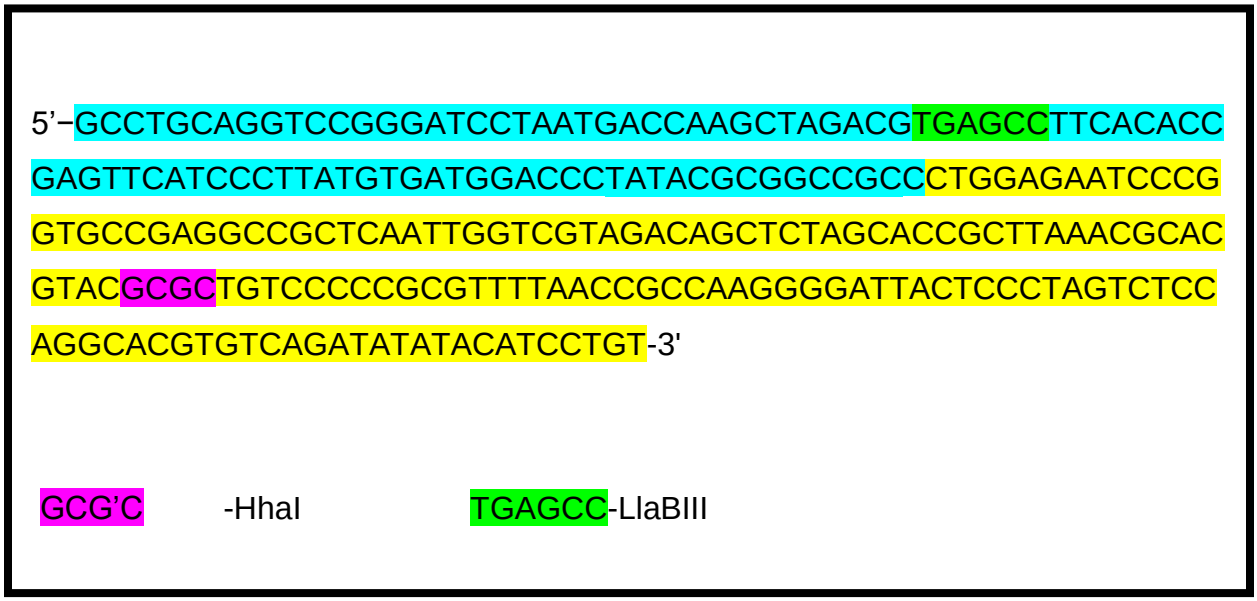


Figure 4: The sequence of DNA substrate that we designed for our biochemical assay showing Type ISP recognition sites LlaBIII in green colour. The image shows the 147p bp 601 sequence in yellow colour which includes a HhaI site (magenta) at its middle.

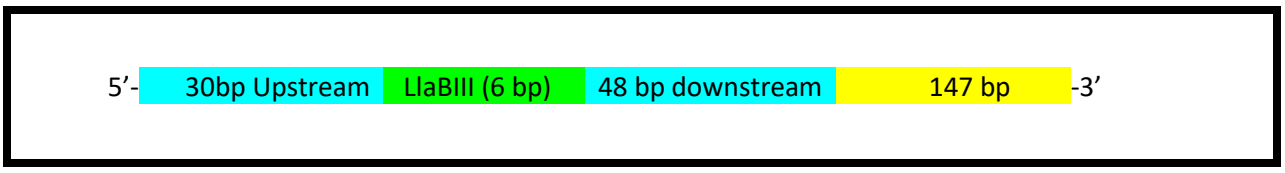


Figure 5: A cartoon of the 238 bp DNA substrate

2.4: Mononucleosome formation

The purified histone octamer that we have is stored in buffer containing 2M NaCl. This high salt concentration is required for the stability of octamer. Sudden decrease in salt would disrupt the octamer and may form dimer, tetramer or aggregates. So, the salt concentration has to be decreased in a slow manner or in a stepwise manner to or below 200mM for the DNA to bind properly to the histone octamer. Once the mononucleosome is formed it is stable at room temperature for few days and at 4 degree for few weeks. We have done both double dialysis and serial dilution method.

In serial dilution, the salt concentration was brought from 2M to 200mM in a step wise manner. 2M →1.5M→1M→800mM→600mM→400mM→200mM with an incubation time of 40 min.

In Double dialysis, the histone octamer which in 2M Nacl and DNA which in elution buffer were mixed in refolding buffer (2M Nacl, 1mMDTT, 10mMTris) and make it 120 microliters. This is transferred into the cap of a 1.5 ml Eppendorf tubes which are made into dialysis buttons with a dialysis membrane having a molecular weight cutoff of 3500 Da. These buttons are again transferred into a dialysis bag having 1M Nacl,1mMDTT,10mM Tris and dialyzed against 1L of B0(1 mM DTT,10 mM Tris, pH 8) overnight

2.5: Biochemical assays designed to check remodeling

The initial biochemical experiments that we planned were binding assays. The idea was to check what happens when ATP is provided to the DNA substrate that is bound to both LlaBIII and histone octamer in 1:1 ratio which is already standardized by titration experiments.

The expected result is if it remodels, we would be able to see free DNA considering that LlaBIII won't rebind and octamer too. But we have to consider the possibility of rebinding of DNA back to the histone octamer. We tried adding 147bp long 601 DNA substrate to prevent rebinding. The DNA was added after 10-15 min, such that all the translocation by

LlaBIII would have already begun. The amount of 601 DNA that has to be added where experimentally standardized. So if remodeling happens we get just LlaBIII bound DNA as shown in the figure below lane g. Since EMSA was showing so many complication like rebinding of histone octamer, standardizing the amount of inhibitor that has to used and limitations in viewing all the markers and super shifts in a 5% native gel led us to stop doing this binding assay. Instead, we chose to use restriction digestion assay to check whether LlaBIII could remove the roadblock.

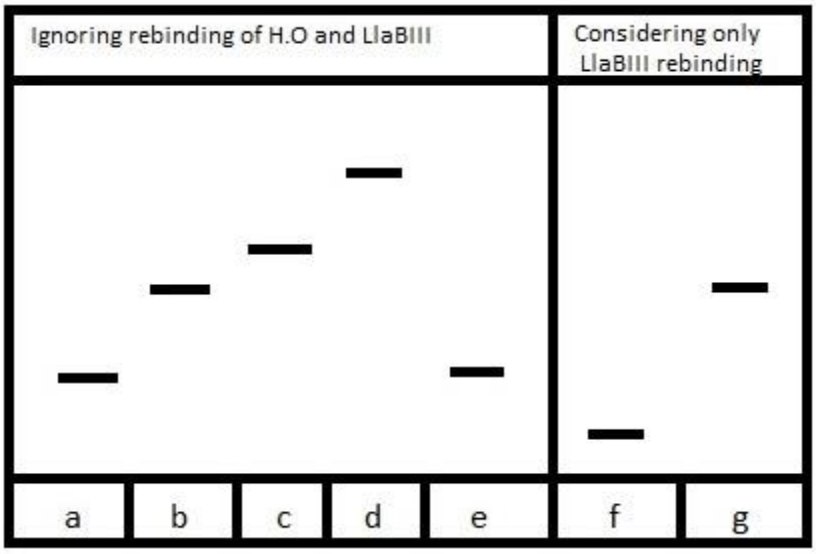


Figure 6: Expected results from binding assay (a)DNA substrate (b)Histone octamer binding to DNA substrate with 1:1 ratio (c)LlaBIII binding to DNA substrate with 1:1 ratio (d)super shift due both histone octamer and LlaBIII binding to the DNA substrate (e)100 % road block removal by LlaBIII ignoring the rebinding of histone octamer and LlaBIII (f)Marker 147 bp 601 DNA which is used as an inhibitor for rebinding of histone octamer (g)100% remodeling considering only LlaBIII rebinding

The DNA substrate that we designed have 147 bp 601 site, where histone octamer is occupied while forming the mononucleosome.147 bp 601 has HhaI site at its middle. HhaI enzyme cannot cleave it if histone octamer is bound. If LlaBIII in presence of ATP removes the roadblock, it will result in the site becoming visible for HhaI to bind and cleave. This will be observed as 238 bp DNA substrate getting converted to two cleaved DNA strands 164 and 74 bp long. SDS and proteinase k treatment are done to the sample before loading to the gel, to avoid rebinding and to see just free DNA. The expected image would look like Figure 7

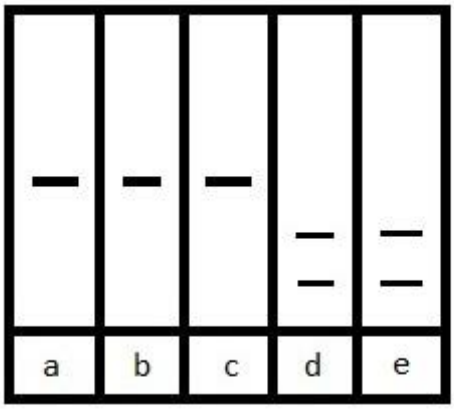


Figure 7: Expected result from cleavage assay. (a)238 bp DNA substrate as marker (b) mononucleosome (c) Mononucleosome bound to LlaBIII without the presence of ATP and HhaI (d) 238 bp DNA substrate in presence of HhaI (e) Mononucleosome bound LlaBIII with the presence of ATP and HhaI treated loaded, considering 100% removal of road blocks.

Results and Discussion

3.1: Expression of H2A, H2B, H3 and H4

IPTG was used for inducing the culture. Induction was done when the OD is in between 0.6 - 0.8. The culture was grown overnight approximately 12 - 16 hrs.5 ml culture was grown initially for expression check later for purification we were growing 2L culture. Expression check was done for all 4 recombinant proteins H2A, H2B, H3 and H4. Both uninduced and induced culture were pelleted down and lysed using lysis buffer (). After sonication was done, then a small amount (20microliter) of induced culture was taken out as sample to be loaded on the SDS-PAGE gel as all cell (AC), remaining were spin down at 13,5000rpm and small fractions(20microliter) of both pellet(PT) and supernatant(SN) were loaded on 15% SDS -PAGE to check whether the protein is more in soluble form or in pellet.

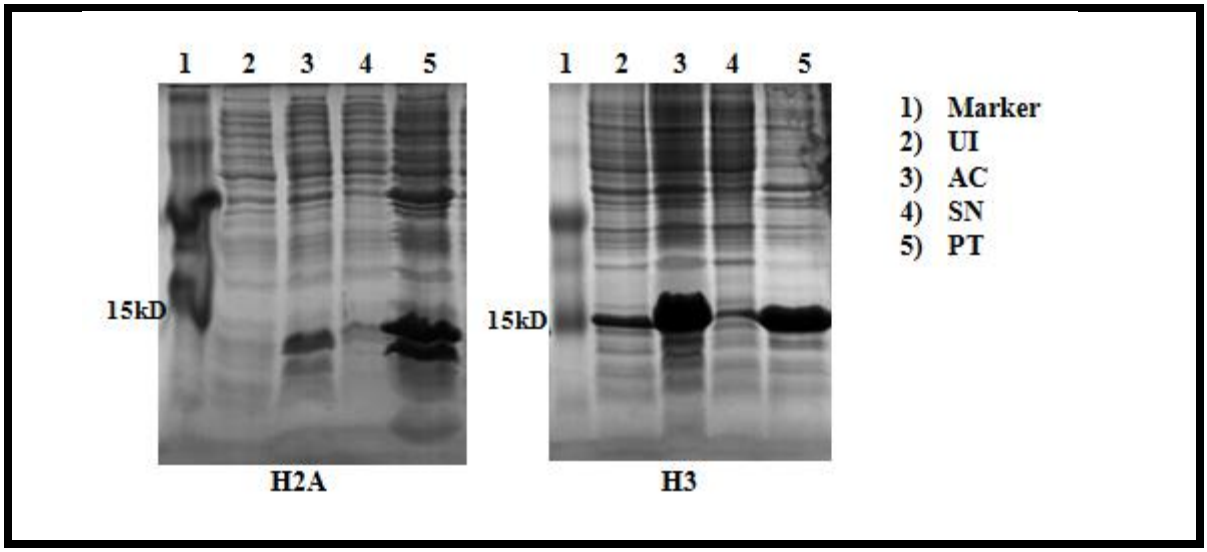


Figure 8:15% SDS PAGE gel showing the expression of H2A and H3 .UI-Uninduced-All cell, SN-Supernatant, P-Pellet. Marker that we have used is of 15kDa.

We observed that most of the protein was going in the pellet. We tried to increase the expression of protein in soluble fraction by optimizing the secondary culture growing temperature, duration and O.D at which we do induction. After adding 7M urea as

denaturing agent to the culture, we did expression check again. We were able to get most of the protein (90%) in soluble form but in denatured form (unfolded state).

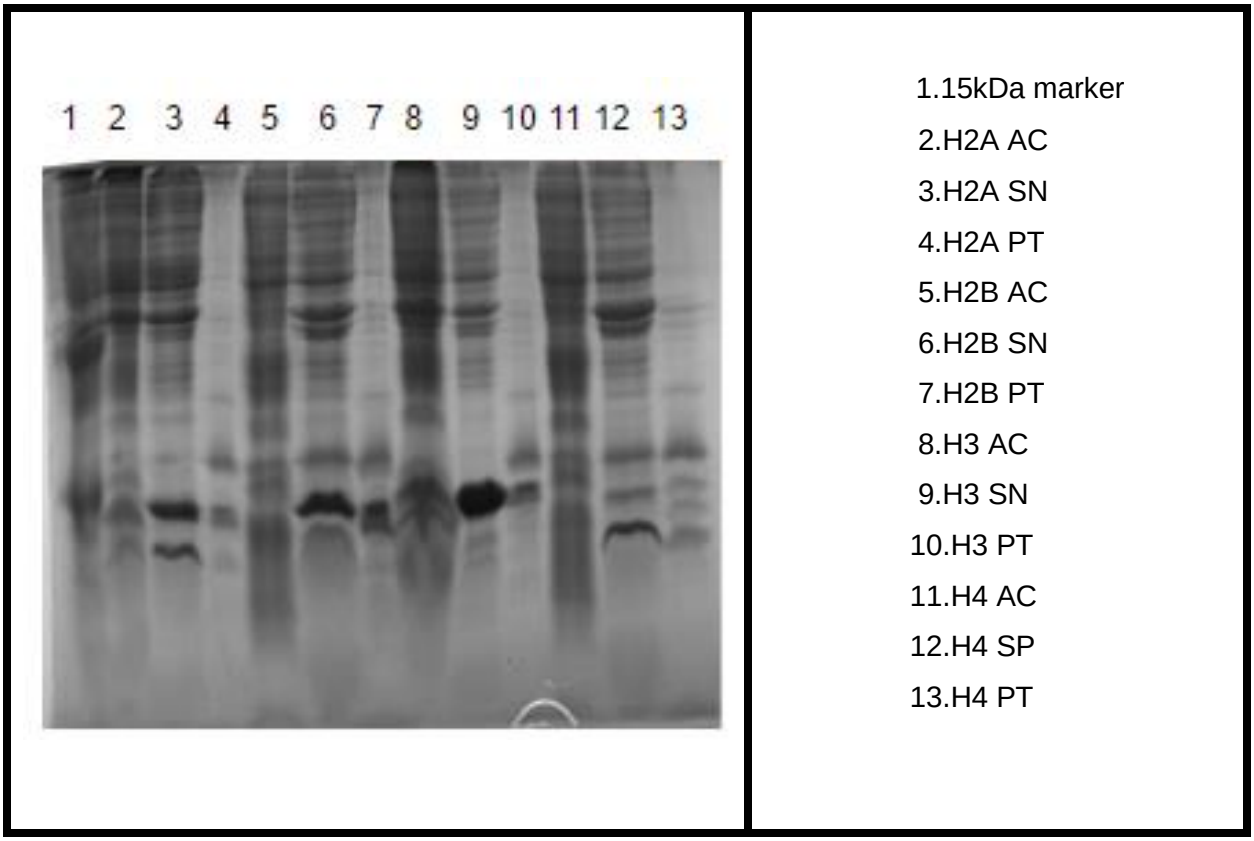


Figure 9:15% SDS-PAGE showing the expression of H2A, H2B, H3 and H4 after adding 7M urea as denaturing agent.AC-All cell, SN-Supernatant, PT-Pellet

3.2: Purification of the histone subunits H2A, H2B, H3 and H4

Ion exchange chromatography was done after getting most of the proteins in soluble fraction by the addition of 7M Urea as denaturation agent. Load is the soluble fraction which is injected or loaded in to the ion exchange columns, Pellet is obtained after ultracentrifugation which is supposed to have less or no recombinant protein. All cell is the sonicated sample which is used for ultracentrifugation. Fractions were collected and checked on a 15% SDS -PAGE. H2A eluted at 580-756 mM NaCl Concentration(6th-9th) (figure A), H2B eluted at 536-624 mM NaCl Concentration(5th-6th) (figure B), H3 eluted at 492-624 mM NaCl Concentration(5th-7th) (figure C), H4 eluted at 536-624 mM NaCl Concentration (5th-6th) (figure D).

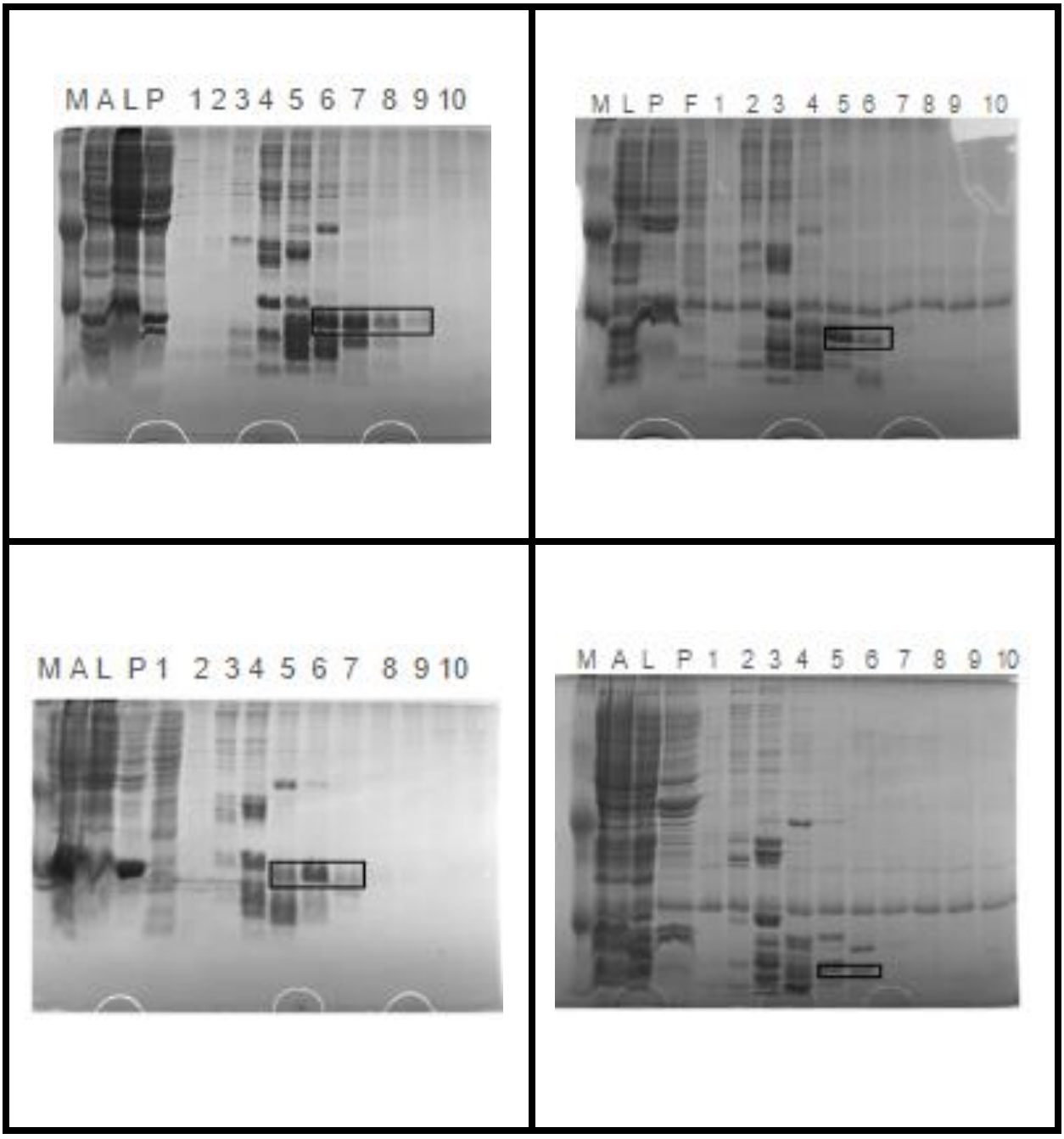


Figure10: 15% SDS PAGE done after the ion exchange chromatography of (A)H2A, (B) H2B, (C) H3 and (D) H4. (M-15kDa marker, A-All cell, L-Load, P-Pellet, F-Flow through, 1-10 -Fractions collected)

Induction at higher O.D(above 0.8) is not favored since it results in overexpression of other proteins too which would result in the higher amount of impurities even after ion exchange which could be removed only in the next step of purification, which is SEC.

3.3: Purification of histone octamers

SEC was done using superdex 200.In 24 ml columns the octamers elute out around 12 ml and in 120 ml column they used elute at around 60 ml. Initial fractions are higher order aggregates, then comes octamers, the shoulder peak is tetrameric contaminants then comes dimers and finally monomers and other small size impurities. If the mixing up of each subunit are not in proper portions the yield of octamer will be less. If the mixing portions are slightly improper it can result in either higher amount of higher order aggregates or dimer etc. The dialysis buffer used is refolding buffer(2L) which has to be changed at least 1 time. Overnight dialysis is required since the histone subunits are in 7M guanidine hydrochloride so that the unfolded protein need enough time to get in properly folded assembled form called histone octamer. Even Though the UV plot gives you in which fractions octamers have eluted, it is better to go with 15% SDS-PAGE gel to check the quality of octamer. A good quality octamer would give the 4 bands in equal stoichiometry which could be resolved better in an 18% gel, since H2A, H2B and H3 are of relatively same size.

SEC using both 120 ml and 24ml was done. Histone octamers eluted at around 60 ml in a 120 ml superdex column and 12 ml in a 24-ml column.

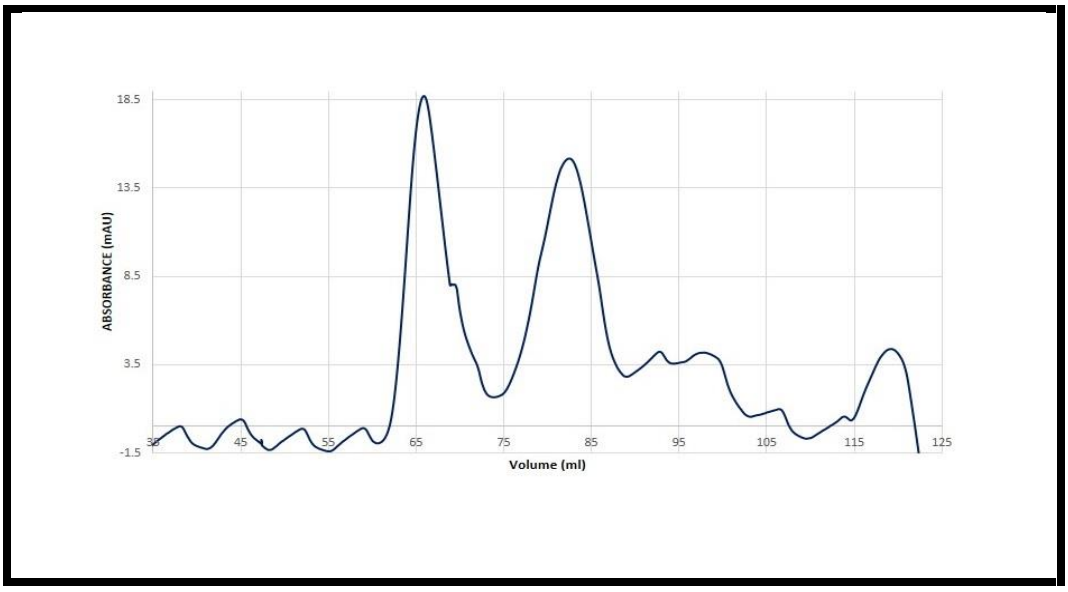


Figure 11: UV plot for superdex 200 120 ml column.

In figure 15, the column used is 120 ml superdex. The first peak that is observed is the octamer and shoulder peak is tetrameric contaminants. The second peak is of H2A -H2B dimers

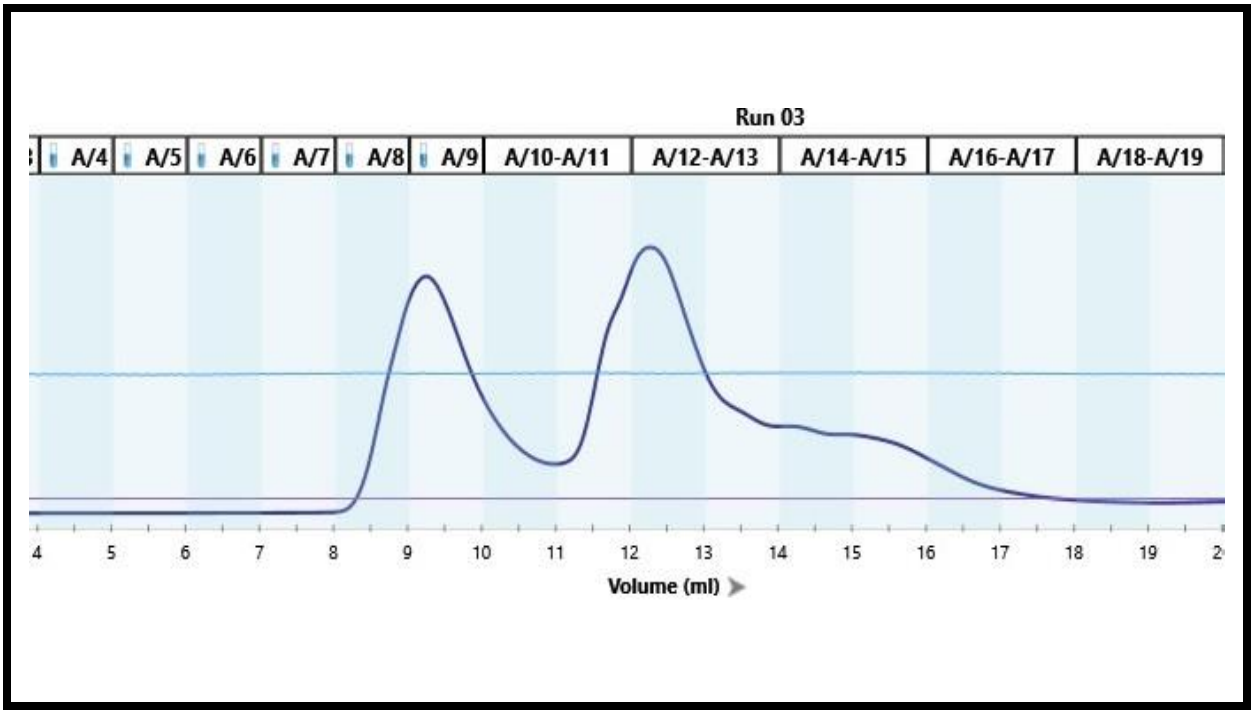


Figure 12: UV plot of superdex 200 24 ml column

In figure 16 the column used is 24 ml one, the histone octamer eluted at around 12.5 ml. The initial peak is of higher order aggregates and the dimer peak is not observed and the shoulder peak observed which of H3-H4 tetramer.

Based on the UV plot we loaded the fractions on a 15% SDS page to gel to check the octamer that we got is of good quality. The bands that we were able to see clearly was 3 since except H4 all other three subunits have almost same molecular weight it is difficult resolve them. The octamers were there in fractions 8-10(11.5-13 ml).

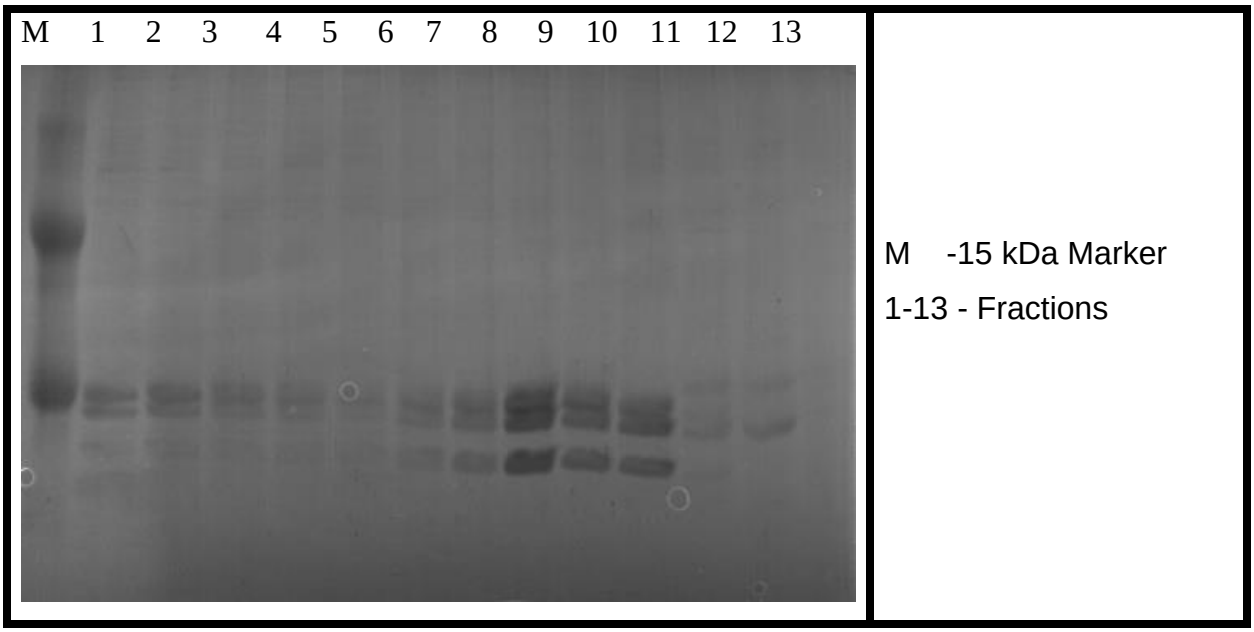
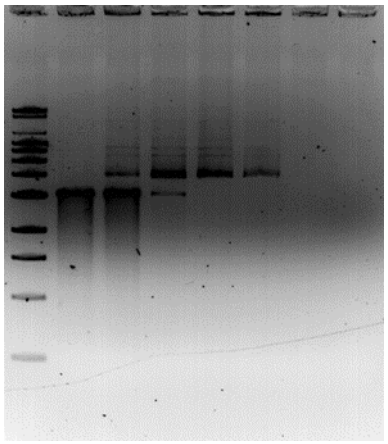


Figure 13:15% SDS PAGE after superdex 200 SEC (24 ml column)

3.4: Mononucleosome formation

Mononucleosome formation was the trickiest part of the project. Concentration measurement at 280 reading using nanodrop wouldn't give the actual concentration. The actual concentration is got by multiplying this 280 reading with the molar extinction coefficient of recombinant histone octamer (for human histone octamer this value is 2). The sudden temperature variations would alter the amount of actual histone octamer so that it is preferred to keep the concentrated stock in 50% glycerol. The histone octamer in 50% glycerol has to be mixed properly by pipetting several times, otherwise the upper layer might have higher concentrations of octamer due to the higher density of glycerol. In 1:1 ratio they used to give single shift which is supposed to be the mononucleosome. In addition, they show multiple shift as we increase the amount of histone octamer (Figure 16, figure 17). The multiple shift could be because of multiple binding of histone octamer. Since to check whether LlaBIII would be able to remove the roadblock is difficult to monitor on a 3kb plasmid, we designed a DNA substrate for our biochemical assays which is mentioned in materials and methods.

A smaller additional shift which is seen in figure 18 when we use 238 bp DNA is because of centrally positioned mononucleosomes. Laterally positioned mononucleosomes is observed to move a little faster than the centrally positioned ones (6). If you further increase the amount of histone octamer the bands start completely vanishing (figure 16, figure 17) which could be because of the formation of soluble aggregates which couldn't enter in a 1% agarose gel if the DNA is 3Kb linearized plasmid having 601 site and in case of smaller substrate (238 bp) they seem to be not entering the 5% native -PAGE. The quality of octamer would also affect the yield of mononucleosome. So, it is advised to check whether the 260/280 reading is 0.6 or below. If it is .6 or below it shows the DNA contaminant is less. We were not able to make complete shift with the 238 DNA substrate that we designed. The results seem to be showing that there is in equilibrium between bound state and unbound state.



lane I -Marker
lane 2 -Linearized 3kb plasmid
lane 3-8 -Increasing concentration of histone octamer.

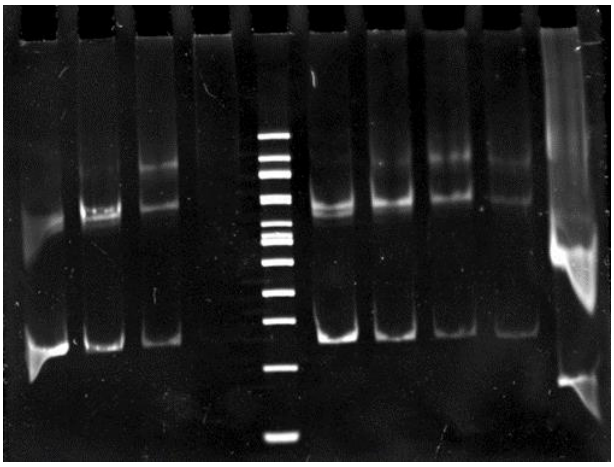
Figure 14: 1% Agarose gel showing the histone octamer binding to 3Kb plasmid (using serial dilution method

The mononucleosome formation could be done by 2 methods as mentioned in the materials and methods and the process of mononucleosome formation requires time decreasing the salt concentration from 2M to 200mM should be done slowly. The 2M Nacl concentration is required for the stability of histone octamers as we decrease the salt

concentration the DNA which is having -ve charge will replace the decrease in Cl- concentration around the histone octamer. Once the concentration of salt is brought to or below 200mM mononucleosome would be formed properly. Once the mononucleosome is formed, they are stable even at room temperature for few days.

3.4.1: Serial dilution method

In serial dilution method 2M NaCl in the storage buffer of histone octamer is diluted to 200mM in a step wise manner(2M->1.5M->1M->800mM->600mM->400mM->200mM). At higher concentration of proteins there seems to be additional shifts and at very high concentrations (double the amount) the yield of mononucleosome is less, all the 3-band intensity doesn't add up to the total in what we see in 1:0.5 or 1:1 ratios. At higher concentrations, they seem to be forming soluble aggregates that won't enter to 6% native page gel. The same pattern is observed both in lane 4 and lane 8 which are of different batch of purified histone octamers

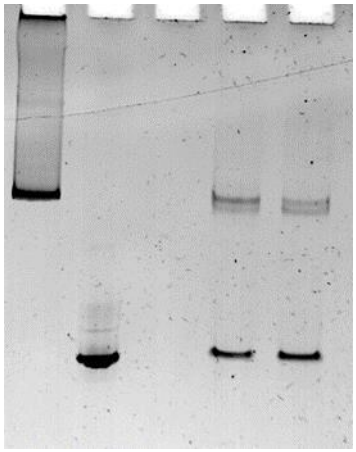


lane 1: DNA: octamer =1:0.5
lane 2: DNA: octamer =1:1
lane 3: DNA: octamer =1:1.5
lane 4: DNA: octamer =1:2
lane 5: ladder
lane 6: DNA: octamer =1:0.5
lane 7: DNA: octamer =1:1
lane 8: DNA: octamer =1:1.5
lane 9: DNA: octamer =1:2
lane 10: marker (145bpmononucleosome)

Figure 15 :6% native PAGE done after the titration of histone octamer against 238bp DNA substrate by serial dilution method

3.4.2: Double dialysis method

The method is as mentioned in materials methods. In figure 22 we were able to see two closer bands as in lane 6 of figure 21.one of the which moves faster could be the laterally positioned mononucleosome and the one moves a little slower is centrally positioned one. Usually dialysis method used to give higher yield of mononucleosome as compared to serial dilution method.



lane 1: 500bp DNA as marker
lane 2: 238 bp DNA substrate
lane 3: (empty)
lane 4: mononucleosome I
lane 5: mononucleosome II

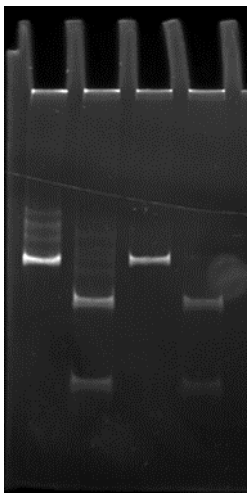
Figure 16: Mononucleosome formed with 238 bp DNA substrate using double dialysis method.

3.5: Remodelling assays

Cleavage assay- I

HhaI site is not accessible in mononucleosome, since the site is occupied by histone octamer. The mononucleosome that we used had some unbound 238 bp DNA. In presence of LlaBIII and ATP there was 100% cleavage showing that HhaI site become accessible for cleavage activity, which shows that LlaBIII could remove the road block. One thing that made us confused was the unbound DNA in mononucleosome that we used for this assay which has not been cleaved at all (lane 3, figure 23), which could be because of excess amount LlaBIII that might have occupied the free DNA, since it can bind nonspecifically too. When you add ATP to mononucleosome in presence of LlaBIII

(lane 4) we were able to see 100% cleavage which shows that in presence of ATP LlaBIII could remove the road block



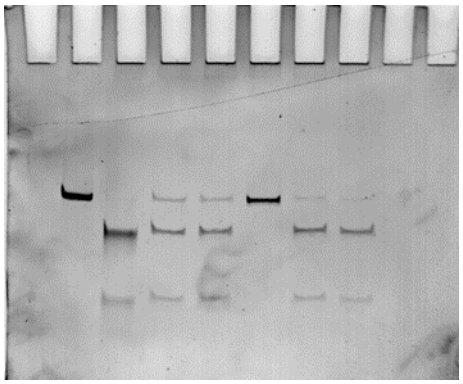
lane 1:238 DNA
lane 2:238 +HhaI
lane 3: mononucleosome+LlaBIII+HhaI -ATP (without ATP)
lane 4: mononucleosome +LlaBIII+HhaI +ATP (with ATP)

Figure 17:6% native PAGE showing the result of cleavage assay-I done on mononucleosome to check whether LlaBIII could remove the roadblock

Remodeling assay- II

The next experiment that we have done also led us to believe that LlaBIII could remove the roadblock.

The amount free DNA remains the same in lane 3 and lane 4 of figure 24. ln lane 5 there is no cleavage at all. It could be that the free DNA could have been bound by LlaBIII non-specifically and hiding HhaI site. This would prevent HhaI from cleaving the DNA. When we compare the amount of free DNA in lane 6 and lane7, we observed that the amount free DNA in lane 7 has gone down when you add ATP again in half way of incubation time. This clearly shows that LlaBIII in presence of ATP displaces mononucleosome and renders it susceptible for restriction digestion by HhaI. As LlaBIII translocate along DNA in presence of ATP, the non-specifically bound LlaBIII, if any, will also get displaced. Hence the masking of HhaI site by non-specific binding of LlaBIII was not observed in presence of ATP. Our next aim was to get the 100% cleavage as in figure 23

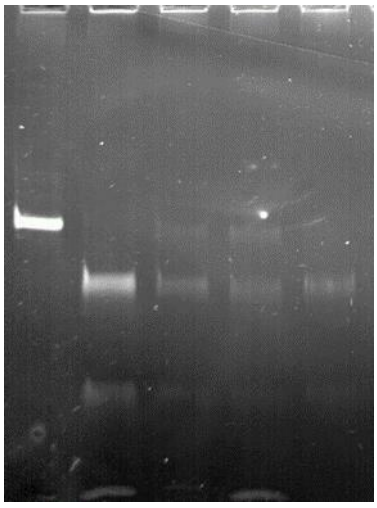


lane 1:238
lane 2:238+HhaI+ATP
lane 3: mononucleosome+HhaI+ATP
lane 4: mononucleosome+HhaI+ATP (addition in half way also)
lane 5: mononucleosome +HhaI+LlaBIII-ATP
lane 6: mononucleosome+HhaI+LlaBIII+ATP
lane7:mononucleosome+HhaI+LlaBIII+ATP (addition in half way too)

Figure 18:6% native PAGE showing the result of cleavage assay -II done on mononucleosome with more controls to check whether LlaBIII could remove the roadblock

Remodelling assay- III

This was the fourth experiment that was done to show LlaBIII could remove the roadblock. This experiment showed almost 100% cleavage of DNA (Figure 19, lane 5).



lane 1:238
lane 2:238+HhaI+ATP
lane 3: Mononucleosome(I)+HhaI+ATP
lane 4: Mononucleosome(I)+HhaI+LlaBIII-ATP
lane 5: Mononucleosome(I)+HhaI+LlaBIII+ATP

Figure 19: 6% native PAGE showing the result of cleavage assay -III

Conclusion

The ATPase motor of the TypeISP RM enzyme LlaBIII has structural similarity to the ATPase motor of the eukaryotic chromatin remodeler Rad 54. Both the motors belong to SF2 helicase family, which made us think whether they have functional similarities. We found that LlaBIII could displace mononucleosomes acting as roadblocks. In future, we plan to repeat this experiment using purified mononucleosome complex lacking free DNA. The biggest problem that we were facing was getting 100% mononucleosome formation. Meticulous measurement of DNA and histone octamer to get 1:1 ratio has to be done. Increasing protein concentration seems to result in many histone octamer bound to DNA, and even higher protein concentration resulted in soluble aggregates. A prokaryotic enzyme working on a eukaryotic system is a big connecting link to evolution. If these enzymes can remodel in vivo too, it would have an impact in genetics and biotechnology and medicine.

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