Cloning, Expression and Characterization of Thermostable Enzymes: Ligase, Lyase and Protease

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

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Certificate of Examination

This is to certify that the dissertation titled **"Cloning, Expression and Characterization of Thermostable Enzymes: Ligase, Lyase and Protease"** submitted by **Ms. Anu Yadav** (Reg. No. MS10092) 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. K. Chattopadhyay Dr. Rachna Chaba Prof. P. Guptasarma

(Supervisor)

Dated: April 24, 2015

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Prof. Purnananda Guptasarma 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.

Anu Yadav

(Candidate)

Dated: April 24, 2015

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.

Prof. P. Guptasarma

(Supervisor)

Acknowledgement

I would like to thank Prof. Purnananda Guptasarma for his valuable guidance and support. His valuable suggestions always helped me to move forward both academically and personally.

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Abstract

Organisms known to thrive in extreme conditions particularly high temperatures have enzymes or proteins with high structure, kinetic and thermal stability. They are more efficient than mesophilies in performing their activity at high temperature. Owing to these qualities inherent in them they are studied for their structure, function, stability, biophysical aspects and their potential applications in industries. In the present thesis, we are reporting the cloning, expression, purification and characterization of three proteins: Arginosuccinate lyase (ASL) and DNA ligase from *Pyrococcus furiosus* and Carboxy Terminal Protease from *Thermotoga maritima*. All the threeproteins under study were found to be highly thermostable. Various biophysical tools namely, circular dichroism, gel filtration, dynamic light scattering and differential scanning calorimetry,etc have been used to characterize the proteins. Also,we attempted to crystallize ASL . Carboxy Terminal Protease(CTP) from *Thermotoga martima* was cloned and protein expression attempts were madebutwe were not able to express it in the *E.coli* strains tried. We studied and understood various properties and nature of some thermophilic proteins.

1. INTRODUCTION

Extremophiles have been known to thrive in extreme environments such as high temperature, pH etc. They are categorised according to the extreme environment in which they grow: thermophiles and hyperthermophiles (high temperature of growth), psychrophiles (low temperature of growth), acidophiles and alkaliphiles (acidic and basic pH), barophiles (high pressure), halophiles (high salt concentration). Scientists are fascinated by them due to their unique mechanism of adaptation and high stability of their proteins. These proteins or enzymes are used for industrial and basic research as they are highly resistant to proteases, detergents and chaotropic agents [11].

Our group mainly works on thermophilic and hyperthermophilic enzymes which have the capability of surviving at moderate to very high temperature. In general, at high temperatures, proteins lacking the necessary adaptations undergo irreversible unfolding, exposing the hydrophobic core and finally aggregation occurs [1]. Hence, proteins from thermophilic organism have several adaptations or mechanisms to maintain their structure and function in extreme environments. Some are believed to be presence of hydrophobic core, ionic interaction, cooperative association, more solvent exposed surface area [1, 2].

1.1 **Proteins**

Proteins are chain of amino acids that fold as primary, secondary, tertiary and quaternary structure to form a biomolecular entity. Primary structure is the sequence of amino acid in the polypeptide chain that is held together by covalent interaction. Secondary structures refer to the α-helix and β-sheets held together by hydrogen bond. The geometry of these structures is defined by ψ and ϕ angles of Ramachandran plot [13]. Tertiary structures are folded 3D secondary structures held together by hydrophobic interactions and quaternary structures are multi-subunit proteins linked by folds and motifs, held by disulphide bond or non-covalent interactions.

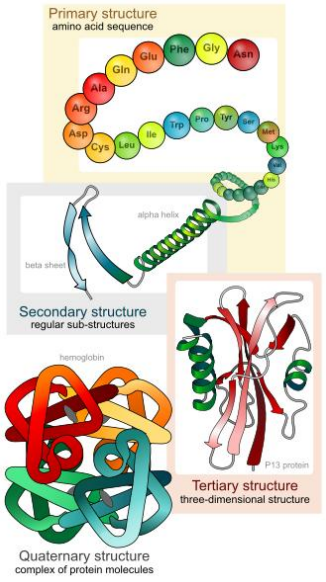


Fig 1.1: Depiction of protein folding into different structures

Cells have a very tight regulation for keeping in check the stability of protein. With thermophilic enzymes, stability is a term related to thermal stability where protein unfolds and refolds reversibly and cooperatively, respectively. It is defined in terms of Gibbs free energy (KJ/mol). The larger and positive Gibbs free energy suggests higher stability of the protein.

$$\Delta G_u = G_u - G_f$$

(ΔG_u= free energy of protein, G_u= Free energy of unfolding, G_f= free energy of folding)

$$\Delta G = \Delta H - T\Delta S$$ (H= Enthalpy (J/Kg); T= Temp (^oC); S= Entropy (J/K))

The stability can be measured in terms of thermodynamic and kinetic stability. Thermostability is the measure of the unfolding and refolding of protein to its original conformation once heated to high temperature and cooled down to room temperature respectively. Kinetic stability, on the other hand, is the measure of how rapidly the protein unfolds. In this case, it is defined as the energy difference between the folded and transition state which determines the rate of unfolding [8].

There is one more aspect of protein stability and that is chemical denaturation which degrades the covalent bonding. At high temperature Asn and Gln are susceptible to deamination, Asp-Xaa peptide bonds were susceptible to hydrolysis, disulphides bonds rupture, and Xaa-Pro peptide bonds undergo cis-trans isomerisation. In hyperthermophilic

proteins disulphide are absent, Asn/Gln content is reduced or Asn/Gln deamidation is reduced presumably be steric constraint making it more stable to high temperature [9].

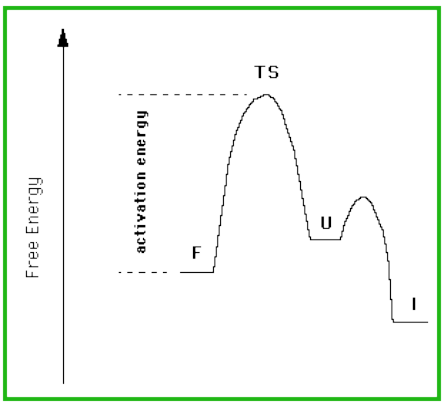


Fig 1.2: Activation energy for protein to unfold

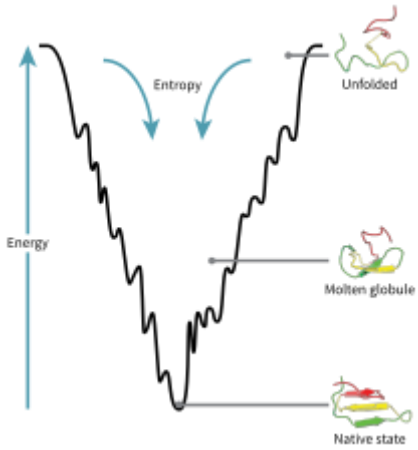


Fig 1.3: Free energy funnel diagram of protein folding [14]

The aim of the present work is heterologous expression, purification and biophysical studies of the three enzymes as listed below

- DNA ligase from *Pyrococcus furiosus*,
- Carboxy Terminal Protease from *Thermotoga maritima*, and
- Argininosuccinate lyase from *Pyrococcus furiosus*

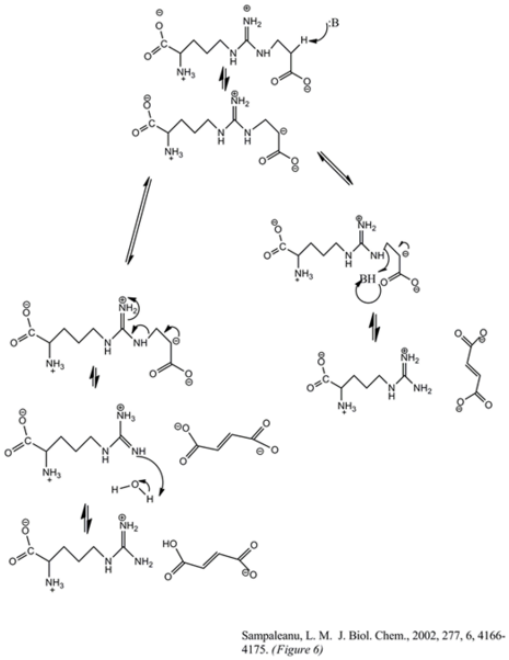
1.2 *Pyrococcus furiosus* Argininosuccinate Lyase (ASL)

Pyrococcus furiosus is an archaea thriving at high temperature of 100^{o}C and pH 7.0. It is one of few organism having enzymes containing tungsten. It has simple respiratory

system which obtains energy by reducing protons to hydrogen gas and uses this energy to create an electrochemical gradient across its cell membrane, thereby driving ATP syntheses. It could be an early respiratory precursor of all respiratory systems of today's higher organism [3].

Argininosuccinate Lyase (ASL) is an important enzyme of urea cycle which is involved in reversible breakdown of argininosuccinate into arginine and fumarate, biosyntheses of arginine and detoxification of ammonia through production of urea. It has a use of intravenous arginine and nitrogen scavenging therapy [10]. Thermostable ASL can be highly useful for industrial applications like producing urea.

It is an enzyme that catalyses the reversible breakdown of argininosuccinate producing the amino acid arginine and dicarboxylic acid fumarate. The reaction of catalyses is E1cb elimination reaction. The base initiates the reaction by deprotonating the carbon adjacent to the arginine, or leaving group.



Sampalcanu, L. M. J. Biol. Chem., 2002, 277, 6, 4166-4175. *(Figure 6)*

Fig 1.4: Mechanism of argininosuccinate lyase activity

1.3 *Pyrococcus furiosus* DNA Ligase

DNA ligase is an enzyme catalyzing phosphodiester-bond formation between adjacent 5'-phosphoryl and 3'-hydroxyl groups at a single strand break in double stranded DNA. This helps in maintaining the genome integrity of an organism [12].

The structure contains three domains: DNA binding domain, adenylation domain and OB-fold domain. It has two bound ligands: Mg ion and AMP. It has a monomeric assembly [12].

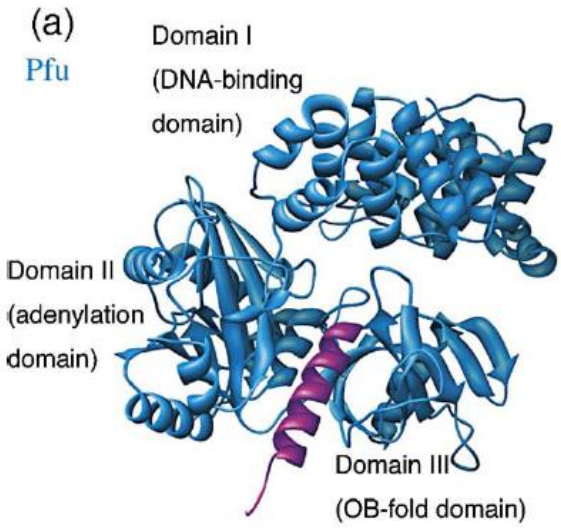


Fig 1.5: Structure of DNA ligase [12]

It forms the covalent phosphodiester bonds between 3' hydroxyl ends of one nucleotide, with 5' phosphate end of another. The reaction proceeds via three sequential nucleotidyl-transfer steps. In the first step, the conserved active-site lysine of the protein is activated by the covalent addition of AMP via a phosphoamide bond to the N atom of the lysine, accompanied by the release of PPi or nicotinamide mononucleotide from the cofactor (ATP or NAD+). In the second step, AMP is transferred from the protein to the 5'-phosphoryl group of the nick on the DNA to form the DNA-adenylate intermediate. In the last step, the phosphodiester bond is formed with the concomitant release of AMP from the DNA-adenylate intermediates [4].

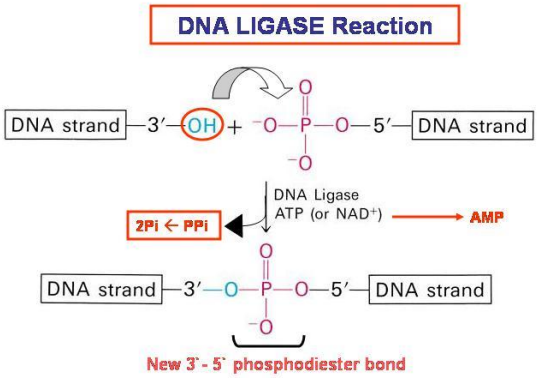


Fig 1.6: Mechanism of DNA ligase activity

Thermostable DNA ligase can be used for its higher efficiency and specificity and less time for the reaction. It can also be used to do research into bioengineering plants suitable in greenhouse on mars.

1.4 *Thermotoga maritima* Carboxy terminal protease (CTP)

Thermotoga maritima is the only thermophilic bacteria known to thrive at 80 ^{o}C which suggests that it could be a potential ancient organism. It shares 24 % of its genome with members of archaea, the highest percentage overlap of any bacteria [5]. This suggests horizontal gene transfer between archaea and ancestors of *T. maritima* and could explain the survival of *T. maritima* at extreme temperature.

Carboxy terminal protease is a serine protease of family S41 which cuts at Ala-Arg after Ala. Its active site is Ser, Lys catalytic dyad [6]. Not much is known about the CTP from *T. maritima* but CTP from plants and *Synechocystis sp.* has been purified and studied. Studies have been done on the D1 protein which is a membrane spanning subunit constitutes the core part of photosystem II [7].

The Serine protease mechanism is shown in the diagram below.

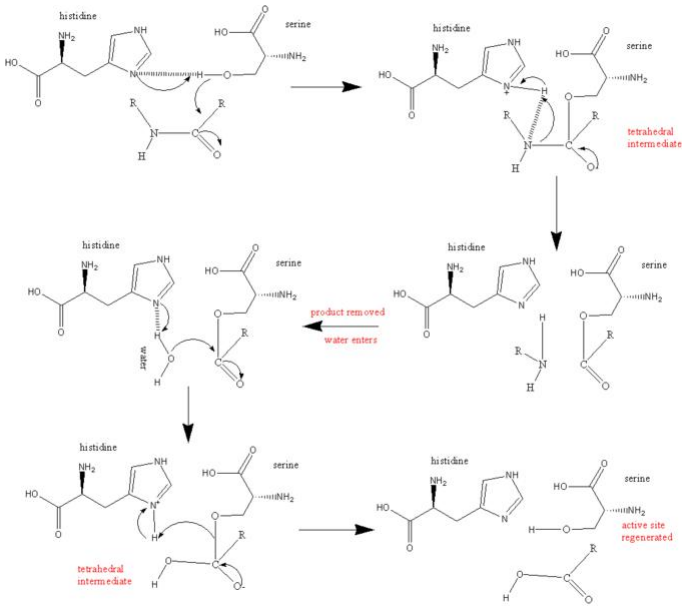


Fig 1.7: Mechanism of serine

CTP can be used for C terminal sequencing and also in industrial applications.

2. MATERIALS

2.1 Chemicals

2.1.1 Media

Luria broth

Component	Amount for LB (1 litre)
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

It was autoclaved (15 psi and 120 ^{o}C) and stored at RT.

LB agar is made by adding 2% agar to above Luria broth solution.

Antibiotics

Antibiotic	Stock concentration (1000 X)
Ampicillin	100 mg/ml in water
Kanamycin	25 mg/ml in water
Tetracycline	12.5 mg/ml in 70 % ethanol
Chloramphenicol	35mg/ ml in methanol

They were sterilized and stored at -20^{o}C. The working concentration was 1X.

dNTP

Stock	Working concentration
100 µM	1µM

Plasmids mini prep

Buffer	Composition	Storage
Buffer P1 (Resuspension buffer)	50 mM Tris-Cl, pH 8.0, 10mM EDTA, 100 µg/ml RNase A	2-8 °C after addition of RNase
Buffer P2 (Lysis buffer)	200 mM NaOH, 1 % SDS (w/v)	15-25 °C
Buffer P3 (Neutralization buffer)	3.0 M potassium acetate, pH 5.5	15-25 °C
Buffer PE (Column Binding /wash buffer)	1.0 M NaCl, 50 mM MOPS, pH 7.0, 70 % ethanol	15-25 °C
Buffer EB (Elution buffer)	10 mM Tris, pH 8.0	15-25 C

2.1.2 Buffers and solution for recombinant DNA work

Buffer for preparation of chemical competent cells

Calcium chloride	60 mM
Glycerol	15 % v/v
PIPES	10 mM

The pH of the buffer is adjusted to 7.0 with NaOH and the final volume is made up as per requirement with deionized water. The solution is sterilised by passing through 0.22 µm filter followed by autoclaving and stored at 4 °C.

6X DNA gel loading buffer (In deionized water)

Bromophenol blue	0.25 %
Glycerol	30 %.

50X TAE

Tris.Cl	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

The pH of the solution is adjusted to 8.0 and deionized water is used to make the final volume to 1 L.

Ethidium bromide stock solution (1% w/v)

Ethidium bromide	0.1 g
Deionized water	10 ml

The stock solution is stored in amber color vial/bottle at 4 °C till further use.

TE buffer (In deionized water)

Tris.Cl (pH 8.0)	10 mM
EDTA	1 mM

2.1.3 Buffers and solutions for detection and analysis of protein

Acrylamide (30.8 % T*, 2.7 % C*)

Acrylamide	30 g
N, N'-Methylene bisacrylamide	0.8 g

Volume made upto 100 ml with deionized water.

Lower Tris (4X), pH 8.8

Tris	18.17 g
10 % SDS	4 ml

Volume made to 100 ml with deionized water after adjusting to pH 8.8 with 6 N HCl.

Upper Tris (4X), pH 6.8

Tris	6.06 g
10 % SDS	4 ml

Volume made to 100 ml with deionized water after adjusting to pH 6.8 with 6 N HCl.

5X Sample loading buffer

Tris. Cl (pH 6.8)	0.15 M
SDS	5 %
Glycerol	25 %
β-mercaptoethanol	12.5 %
Bromophenol blue	0.06

Volume made to 10 ml with deionized water.

Laemmli buffer (Laemmli, 1970)

Tris buffer	3.0 g
SDS	1.0 g
Glycine	14.4 g

Volume made upto 1 L with deionized water.

Gel staining solution

Methanol	40 %
Glacial acetic acid	10 %
Coomassie Brilliant Blue R-250	0.1 %
Deionized water	50

Gel destaining solution

Methanol	40 %
Glacial acetic acid	10 %
Deionized water	50

SDS PAGE composition

Resolving gel (15%)

Composition	Volume used
Lower tris	2.5ml
Acrylamide	5ml
Water(deionized)	2.4ml
APS	50ul
TEMED	10ul

Stacking gel (5%)

Composition	Volume used
Upper tris	0.5ml
Acrylamide	0.333 ml
Water	1.167ml
APS	12.5ul
TEMED	5ul

The composition of the stacking and resolving gels is the same as specified by Sambrook *et. al.* (1989) and Sambrook and Russel (2001).

* T – Total % concentration of both monomers (acrylamide and bis-acrylamide),

C - % concentration of crosslinker relative to the total concentration T.

2.1.4 Buffers and solutions for protein purification

Denaturing purification of 6X His-tagged proteins:

Lysis Buffer, B

Urea/GdnCl	8 M/6M

NaH_2PO_4	0.1 M
Tris.Cl	0.01 M

pH is adjusted to 8.0 with NaOH

Wash Buffer, C

Urea/GdnCl	8 M/6M
NaH_2PO_4	0.1 M
Tris.Cl	0.01 M

pH is adjusted to 6.3 with HCl

Elution Buffer, D

Urea/GdnCl	8 M/6M
NaH_2PO_4	0.1 M
Tris.Cl	0.01 M

pH is adjusted to 5.9 with HCl

Elution Buffer, E

Urea/GdnCl	8 M/6M
NaH_2PO_4	0.1 M
Tris-Cl	0.01 M

pH is adjusted to 4.5 with HCl

Non-denaturing/native purification of 6X His-tagged proteins:

Non-denaturing/Native Lysis Buffer

NaH_2PO_4, pH 8.0	0.05 M
NaCl	0.3 M
Imidazole	0.01 M

Non-denaturing/Native wash Buffer

NaH_2PO_4, pH 8.0	0.05 M
NaCl	0.3 M
Imidazole	0.02 M

Non-denaturing/ Native Elution Buffer

NaH_2PO_4, pH 8.0	0.05 M
NaCl	0.3 M
Imidazole	0.25

2.2 Plasmids

The gene was to be inserted into a plasmid compatible for transformation in *E.coli*. These plasmids have origin of replication, antibiotic selection, compatible promoter and multiple cloning sites.

- pET23a: size is 3.6 kbp, containing a T7 promoter and ampicillin resistance gene as selection marker. The vector is used for expression of proteins fused with a 6X His tag at C-terminal end.
- pQE30: size is 3.4 kbp, containing a T5 promoter and ampicillin resistance gene as selection marker. This is used for expression of 6X N-terminal His tag proteins.

2.3 Cloning

Primers

Stock	Working concentration
100µM	1µM

Bacterial strain used:

Strains	**Relevant genotype or phenotype**
***E. coli* BL21(DE3)**	*F^- ompT gal dcm lon hsdS$_B$ (r_B^- m_B^-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])* ▪ an *E. coli* B strain with DE3, a λ prophage carrying the T7 RNA polymerase gene and $lacI^q$. ▪ lac UV5 promoter inducible by IPTG. ▪ transformed plasmids containing T7 promoter driven expression were repressed until IPTG induction of T7 RNA polymerase from a lac promoter. ▪ derived from B834 (Wood, 1966) by transducing to Met^+.
***E. coli* XL1 Blue**	*endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB$^+$ $lacI^q$ Δ(lacZ)M15] hsdR17(r_K^- m_K^+)* ▪ derived from *E. coli* K 12 strain ▪ nalidixic acid resistant ▪ tetracycline resistant (carried on the F plasmid)
***E.coli* Rossetta**	BL21 (DE3) derivative, Expresses six rare tRNAs; **AGG/AGA** (arginine), **CGG** (arginine), **AUA** (isoleucine), **CUA** (leucine), **CCC** (proline), and **GGA** (glycine)

3. METHODS

3.1 Cloning: It is the process of inserting the gene of interest into a compatible vector to express the protein of interest. It proceeds via the following step:

- Amplification of gene: The gene of interest was amplified using genomic DNA by utilizing the primers.
- Digestion of gene and vector: The respective gene and vector was digested using restriction enzymes at 37^{o}c for half hour.
- Ligation: The digested vector and gene was ligated using T4 DNA ligase at 25^{o}C for 3 hours.
- Transformation: The ligated product was transformed in respective strains of *E.coli* (chemical competent cells) and plated on LB plates with respective selection of antibiotics.

3.2 PCR: Polymerase chain reaction is a process for amplification of gene using DNA polymerase, dNTPs (dATP, dCTP, dGTP, dTTP) and set of primers that were complimentary to gene. The DNA is denatured first; primers bind to the denatured strands through the complementary sequence and then DNA polymerase act and increase the length of gene by adding complementary dNTP to the strand. For amplification, 1-2 ng of genomic DNA was used in final reaction volume of 25ul, containing 500 uM of dNTP, 1.25 uM of each primer, 1X thermopol buffer and 0.5 units of DNA polymerase.

S.No	Steps	Taq Pol (NEB)	Deep Vent/Vent (NEB)	Phusion (Thermo)	
1.	Initial denaturation	95^{o}C, 30 sec	95 °C, 5 min.	98 °C, 5 min.	25-30 cycles
2.	Denaturation	95^{o}C,30 sec	95 °C, 30 sec	95 °C, 10-15 sec	
3.	Annealing	45 sec (temp depends on the T_m of primers)	30 sec (temp depends on the T_m of primers)	20 sec (temp depends on the T_m of primers)	
4.	Extension	68^{o}C, 2 min (time depend upon length	72 °C, 30sec-1 min/kb	72 °C, 30sec/kb	

		of gene)			
5.	Final extension	68^{o}C, 5 min	72 °C, 5 min.	72 C, 5 min	

3.3 Extraction of DNA from agarose gel: The PCR product was loaded onto agarose gel with markers to check for the correct band. The right size band was extracted and purified from the gel using gel extraction kit (Qiagen, Germany) using the following steps:

- Gel was solubilised at 50^{o}C by adding 3:1 ratio of QG buffer to reaction vol.
- 100 ul of isoproponol was added and dissolved product was loaded onto spin column to allow adsorption of DNA onto silica gel matrix.
- The impurities were washed with ethanol containing PE buffer
- DNA was finally eluted with 20 ul of deionized water

3.4 Digestion: The isolated vector and gene was quantified using Nanodrop with absorbance at 260nm. They were digested with specific restriction endonuclease enzymes in their respective buffers. Digestion was carried out at 37^{o}C for 30min (200ng of Gene and 1ug of plasmid) with fast digest enzymes and 5hrs (5-10 U of enzymes/ ug DNA) with NEB enzymes. After digestion, Gene was PCR purified and Plasmid was agarose gel extracted.

3.5 Ligation: Digested and purified plasmid and Gene were set up for cohesive end ligation using T4 DNA ligase or Quick ligase (NEB) in 1X buffer for 20ul of reaction volume. Various ratio of gene: vector was used i.e. 3:1, 5:1, and 7:1. Ligation reaction was set up for 3 hours at 25^{o}C in case of T4 DNA ligase and 25^{o}C for 15 min in case of Quick ligase.

For a particular amount of digested vector (50ng), amount of digested PCR product is calculated by the following formula:

Amount of gene (ng) =

$$\frac{\text{Amt. of digested vector (ng) x molar ratio (gene: vector, 3:1) x Size of gene (bp)}}{\text{Plasmid size (bp)}}$$

3.6 Transformation: After ligation, the product was transformed into cloning host cell. The protocol is as follow:

- The chemically competent cells were thawed on ice for 15min
- Ligation mixture was added into competent cells and kept for 30 mins on ice
- Heat shock was given to the mixture for 90sec and kept on ice for 2 mins.
- 900 ul of LB was added into the mixture and kept at 37^{o}C for 1 hour to grow.
- Mixture is plated onto LB plates having suitable selection and kept at 37^{o}C for overnight incubation.

3.7 Preparation of competent cells: The chemical competent cells were prepared by following the protocol

A single colony of E.Coli was inoculated in LB media and grown to saturation

The culture was reinoculated into 100ml fresh LB medium at a dilution of 1:100 and grown to early log phase (A_{600} of 0.3-0.4).

The cells were chilled on ice for 15min, centrifuged at 5000x g for 15min at 4^{o}C in prechilled centrifuge tubes. Cells were kept on ice at all subsequent steps.

The supernatant was discarded and cells were resuspended in 20 ml of ice cold CaCl2 solution (deionised and autoclaved) after which they were again centrifuged as in step 3.

The supernatant was again discarded and step 4 repeated.

The cell pellet obtained in step 5 is resuspended in 8 ml of ice-cold CaCl2 solution.

Finally aliquots of 80 or 100 µl were made from the suspension obtained above and these were used immediately or stored at –80°C till further use.

3.8 Plasmid purification: Plasmid DNA was purified using the Qiagen miniprep kit. 5ml overgrown culture (overnight incubation at 37^{o}C) was pelleted at 13000 rpm. The supernatant was discarded and 250ul of P1 buffer (resuspension buffer) was added and pellets were resuspended. 250 ul of P2 buffer (lysis buffer) was added and incubated for 1-2mins. 350ul of N3 buffer (neutralizing buffer) was added. The solution was centrifuged for 10mins at 13000rpm. The supernatant was loaded onto spin column and centrifuge for 60 sec. Then the flow through was discarded and column was washed with 750ul of wash buffer (PE buffer) and spun for 1 min. An

empty spun of 2 min was given and the plasmid was finally eluted with deionized water.

3.9 Clone screening: Positive clones were screened by colony PCR using vector specific primers, isolating the plasmids and digestion check for correct size bands and finally with DNA sequencing.

3.10 Expression of proteins in *E.coli*: Gene cloned into pQE30 were transformed into XL1B and gene cloned into pET23a was transformed into BL21 (DE3)/Rossetta. These are due to promoter compatibility of BL21 with pET23a and not with pQE30 plasmid. XL1B is both a cloning and expression host. Overnight cultures were inoculated to a final concentration of 1% in LB containing appropriate antibiotics and grown to OD_{600} of 0.6-0.7 at 37^{o}C with shaking at 220rpm. The culture was induced with 1mM IPTG and grown for further 5 hrs. 1ml of both uninduced and induced samples were run on SDS PAGE by adding 50ul of loading dye and boiled at 99^{o}C and analysed for the protein expression.

3.11 Glycerol stock: For glycerol stack preparation, 1500ul of overnight culture was added with 500ul of 60% glycerol and stored in -80^{o}C.

3.12 Denaturing purification: After inducing the culture as explained above, the cells were harvested down at 8000 rpm for 10 mins. The harvested cells were resuspended in buffer B (lysis buffer having urea, pH 8) of 2-5ml per gram wet weight of pellet. The solution was sonicated to lyse the cells. Overnight incubated at RT. Then the cell debris was isolated from the supernatant by centrifuging at 12000 rpm for 1 hour. The supernatant was passed through the Ni-NTA column. Washed with 4 ml of buffer C (pH 6.3) and eluted with Buffer E (pH 4.5, pH based elution).

3.13 Native purification: After inducing the culture as explained above, the cells were pellet down at 8000 rpm for 10 mins. The harvested cells were resuspended in lysis buffer (pH 8) of 2-5ml per gram wet weight of pellet. Lysozyme was added according to 1mg/ml vol of lysis buffer. The solution was sonicated to lyse the cells and heated to 80^{o}C for ½ hrs. Then the cells were incubated overnight incubated at RT. The cell debris was isolated from the supernatant by centrifuging at 12000 rpm for 1 hour. The supernatant was passed through the Ni-NTA column, washed with 20 ml of wash buffer (20-40 mM imidazole) and eluted with elution buffer (250mM of imidazole).

3.14 PMF for protein identification: Trypsin (proteomics grade) solution (Sigma catalogue no. T6567)

The lyophilized product was reconstituted in 1 mM HCl at the correct concentration. For solution digests, trypsin was prepared in 1 mM HCl at a concentration of 1 μg/ml (20 μl of 1 mM HCl for a 20 μg vial). This results in a solution containing 1 mg/ml trypsin, pH 3.0. For in-gel digestion, a solution is prepared by adding 100 μl of 1 mM HCl to one vial of trypsin. The vial is mixed briefly to ensure dissolution of the trypsin, and 900 μl of 40 Mm ammonium bicarbonate in 9% acetonitrile is added to the vial and mixed. The final concentration of trypsin is 20 μg/ml.

In-gel digestion

1. The band of interest is excised from a 1D gel using a scalpel or razor blade, taking care to include only stained gel. The gel piece is lifted out using clean flat-nosed tweezers.

2. The gel piece is placed in a siliconized Eppendorf tube or equivalent. A siliconized tube reduces binding of peptides to the tube surface. The tube is prewashed with 100 μl of a 0.1%trifluoroacetic acid in 50% acetonitrile solution and then allowed to dry before use, to remove any contaminating substances.

3. The gel piece is covered with 200 μl of 200 mM ammonium bicarbonate with 40% acetonitrile) and incubated at 37 °C for 30 minutes. The solution is then removed and discarded from the tube.

4. Step 3 is repeated one more time.

5. The gel piece is dried in a rotary vaccum centrifuge for approximately 15 to 30 minutes.

6. 20 μl (0.4 μg of trypsin) of trypsin solution prepared for in-gel digestion is added to the gel sample.

7. 50 μl of 40 mM ammonium bicarbonate in 9% acetonitrile solution is added to the gel sample.

8. The gel piece is firmly established at the bottom of the tube and covered with liquid.

9. The immersed gel piece is incubated for 4 hours, or overnight, at 37 °C.

10. After the incubation, the liquid is removed from the gel piece and transfered to a new labelled tube. This liquid contains the extracted tryptic peptides. If MALDI analysis is to be performed at this step, acidification with TFA prior to matrix addition could be performed.

11. 50 µl of a 0.1% trifluoroacetic acid in 50% acetonitrile solution is added to the gel piece and incubated for 30 minutes at 37 °C. Note: This extraction step only increases the peptide yield by about 5%.

12. The 0.1% trifluoroacetic acid in 50% acetonitrile solution is removed and combined with the liquid from Step 10.

13. The combined sample solution from Step 12 is ready for MALDI-MS analysis.

3.15 UV-Vis absorption spectroscopy: The protein concentration was measured using Cary50 UV-Vis spectrophotometer. The absorption reading was taken at 280nm and then calculating the concentration using the formula: A=ECL (A=absorption, E= extinction coefficient, C=concentration of protein and L= path length).

3.16 FPLC: Fast protein liquid chromatography is used to separate the proteins based on molecular weight. This was done using GE's Akta purifier system. The column and loop was washed thoroughly and equilibrated with the buffer used. Column was used based on the size of protein. The elution profile depends on the hydrodynamic radius of protein and gives information about the multimeric state of protein.

3.17 Fluorescence spectroscopy: The emission spectra of proteins were collected in Cary eclipse fluorimeter by exciting at 295nm and collecting the spectra at 300-400nm. The slit width was 5nm and scan speed at medium.

3.18 Circular dichroism spectroscopy: Far UV CD spectra in the range of 250-200nm to see the secondary structures of proteins. The α-helix corresponds to a dip at 208 and 220 nm and β sheet at 214-18 nm. The path length of cuvette was 0.1cm. Raw ellipticity was converted to Mean residue ellipticity by following formula:

$$[\boldsymbol{\theta}] = \frac{\boldsymbol{\theta obs}\ (\boldsymbol{in\ mdeg}) \boldsymbol{x}\ \mathbf{100}\ \boldsymbol{x}\ \boldsymbol{MRW}}{\mathbf{1000}\ \boldsymbol{x}\ \boldsymbol{concentration}\ (\boldsymbol{mg/ml})\ \boldsymbol{x}\ \boldsymbol{path\ length}\ (\boldsymbol{cm})}$$

Where, MRW = Mean Residue Weight (Total molecular weight of the protein / Total number of amino acids), [θ] = Mean Residue Ellipticity and θ_{obs} = Raw Ellipticity.

3.19 DLS: Dynamic light scattering was done using single-angle scatter monitoring of 824nm lazer radiation. Hydrodynamic radius distribution versus percent scattering and percent mass data was collected.

3.20 DSC: Differential scanning calorimeter is used to screen the unfolding state of protein. 0.5-1 mg/ml of degas protein is used. The baseline was set up using the buffer-buffer scan and then the protein was loaded. 20-25 scans were given with scan rate of 90 for heating and 60 for cooling and temperature range 20-100^{o}C.

3.21 FTIR: The infra-red spectrum was collected to get an idea of secondary structure in a protein. Dialysed and concentrated protein (2-3mg/ml) was used for the study. Two regions are usually used for studying i.e. Amide I having range of 1500-1600 cm^{-1} and corresponds to absorption of C=O bond while amide II bands with 1600-1700 cm^{-1} range corresponds to N-H and C-N stretch absorption. Antiparallel β sheet has a peak at 1615-1637 cm^{-1}, parallel β sheet at 1640 cm^{-1}, α helix at 1652 cm^{-1} in amide I and 1548 cm^{-1} in amide II and β turns at 1680 cm^{-1}.

3.22 Crystallization: It is method of forming the crystals by supersaturating the solution. The molecules arranges in a repeated array held by non covalent bonds. The widely used method of protein crystallization is vapour diffusion method. In this, a droplet of purified protein, buffer and precipitant are allowed to equilibrate with a larger reservoir of buffers and precipitant in higher concentrations. As the protein and precipitant concentration increases in the drop, the crystals formed.

Sitting drop method of crystallization was done. Different mixtures of PEG, salt and buffer were used with different ratio of protein to buffer. Buffers were made according to given concentrations in Qiagen,PEG suite I and II. A very high concentration of protein (13mg/ml) was used. The plates were kept at 20^{o}C for 4-7 days.

4. Results

4.1 ARGININOSUCCINATE LYASE (ASL)

The clone for PfuASL was already present in the lab in pET23a vector between Nde1 and Xho 1 restriction sites having C-terminal His-tag. The clone was expressed in BL21 (DE3) strain of *E.coli*. The cloned was digested at Nde1 and Xho 1 to check for the insert.

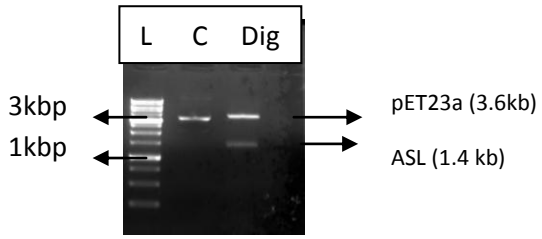
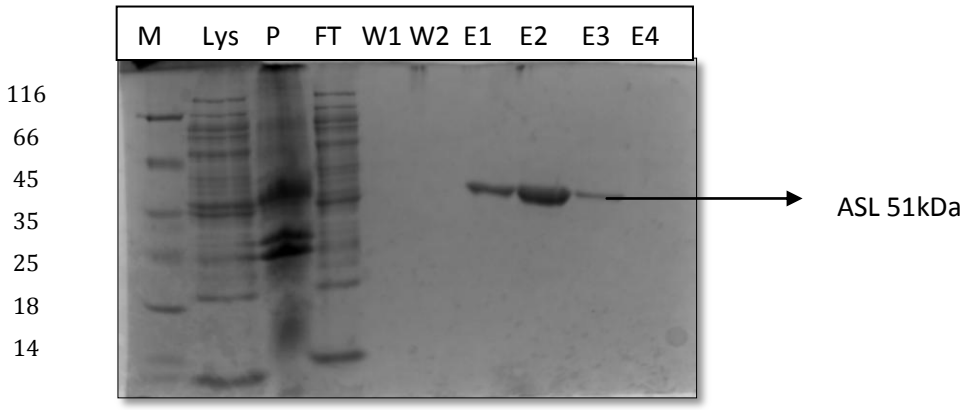


Fig 4.1: Digestion of Cloned Plasmid

4.1.1 Purification of ASL was done under native conditions using Ni-NTA chromatography.



M: marker, Lys: lysate, P: pellet, FT: flow through, W: wash with 10mM and 40mM imidazole, E: elution with 240mM imidazole

Fig 4.2: SDS PAGE of purified ASL

The eluted protein was subjected to dialysis in 20mM Tris and 50mM NaCl at pH 8 to remove imidazole for further analysis.

The correct size band was obtained and I did further analysis with this protein. But after prolonged incubation, **degradation** was seen in protein.

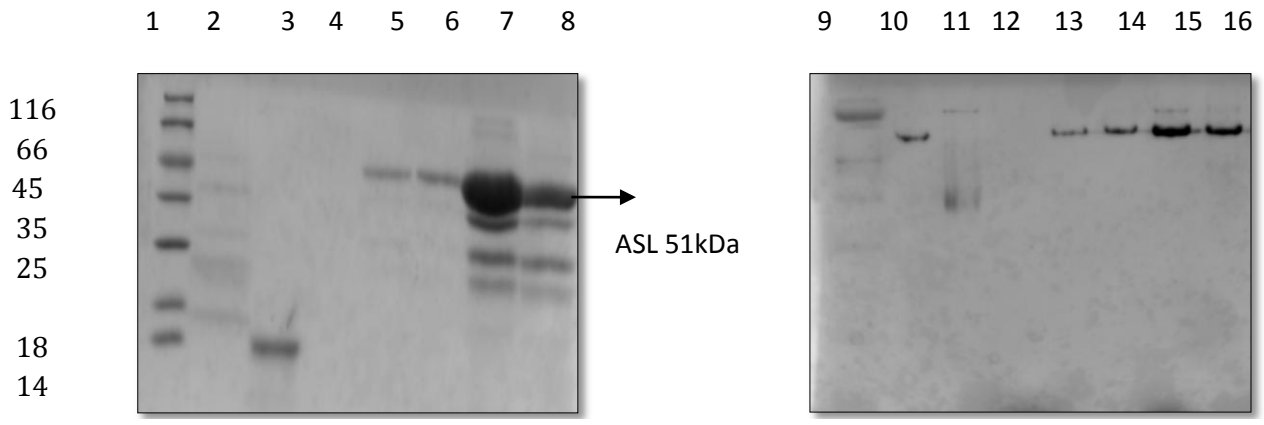


Fig 4.3: The 1-8 lanes show proteins in SDS PAGE and 9-16 in Native PAGE. The above gel shows protein after prolonged incubation

Lanes in above gel:

1 and 9 - Marker
2 and 10 - 40days old protein
3 and 11 - 30 days old protein
4 and 12 - 30 days old protein that was heat purified
5 and 13 - 10 days old protein with 0.3mg/ml concentration
6 and 14 - 10 days old protein with 0.3mg/ml that was heat purified
7 and 15 - 10 days old protein with 3.8mg/ml that was heat purified
8 and 16 - 10 days old protein with 3.8mg/ml

- This shows that although the protein degrades after prolonged incubation and that does not stop even after heating, the protein remain associated as seen in Native PAGE. Although we cannot deduce the size of protein from the Native PAGE as we don't have the markers, we can see that the bands are intact. The intensity is different in different lane due to different concentration.

To stop the degradation, the protein was purified by heating at 70 ^{o}C for half hour, treated with PMSF 5mM concentration, 1mM EDTA and also with Protease inhibitor cocktail.

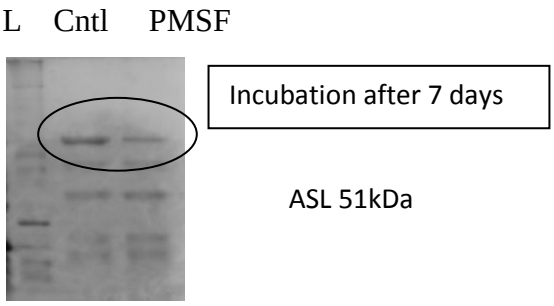
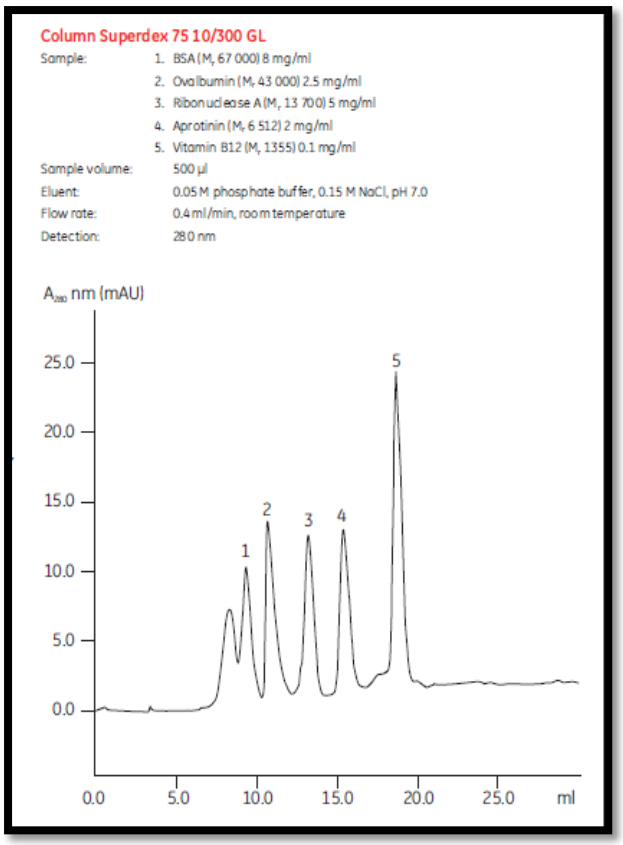
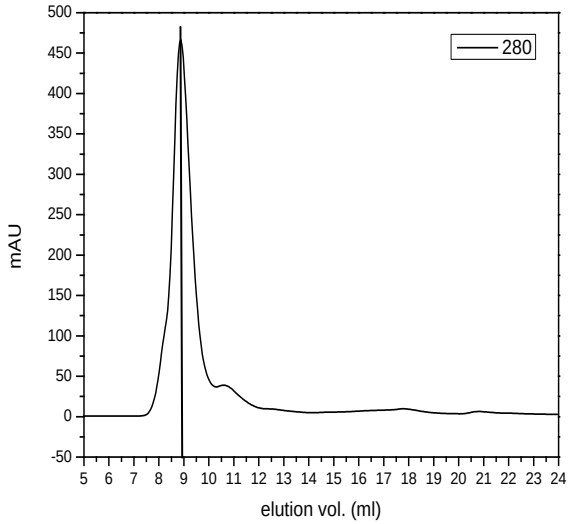


Fig 4.4: SDS PAGE of PMSF treated protein

But nothing was successful to prevent the degradation.

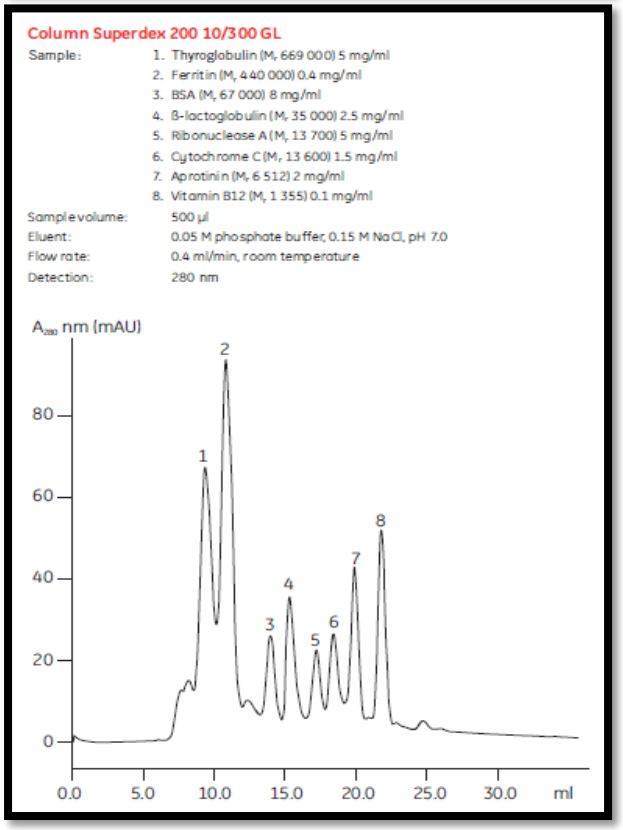
For further analysis of oligomeric state of protein and to separate these bands, **FPLC** was done. Shown below are the standard curves for gel filtration chromatography and the chromatograms of the protein run on Superdex 75 and Superdex 200 column in the 50mM NaH_2PO_4, 300mM NaCl at pH 7 and 20mM Tris and 50mM NaCl at pH 8 buffers respectively. Figure 4.5 shows that the protein is present as a dimer in solution.

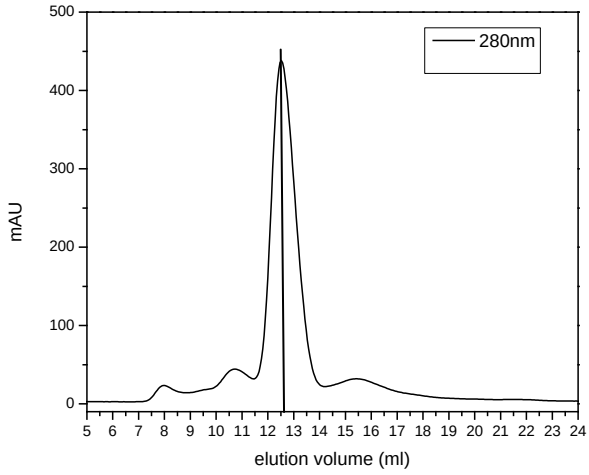




Superdex 75; Protein concentration: 2.5mg

Buffer 50mM NaH_2PO_4, 300mM NaCl at pH 7





Superdex 200; Protein concentration: 0.8 mg/ml

Buffer 20mM Tris and 50mM NaCl at pH 8

Fig 4.5: Gel filtration of ASL

- By comparing with standard graphs, protein was getting eluted as dimer.

4.1.2 Structural analysis

To assess the secondary structure content in protein, Far UV CD spectrum and FTIR spectrum of the protein was recorded. Figure 4.6 represents the CD spectrum of the protein and depicts that the protein is mostly alpha helical in nature as can be seen in the dip at 208 and 220 nm in CD and a peak at 1652cm-1 and 1548 cm-1 in FTIR spectrum (Figure 4.7).

Circular Dichroism (CD)

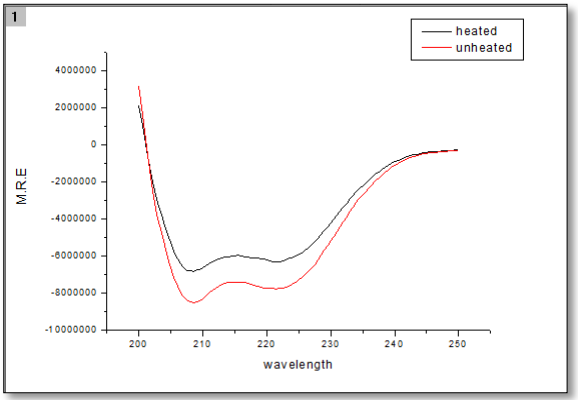


Fig 4.6: CD of ASL

FTIR

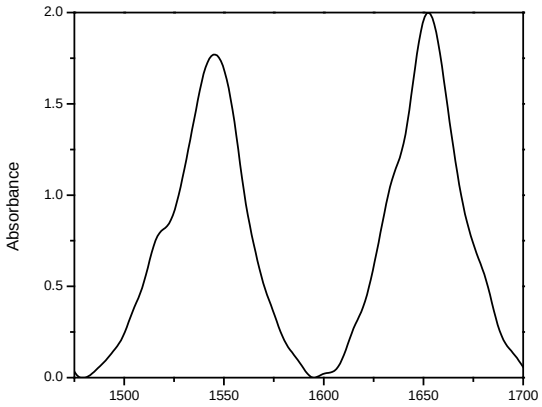


Fig 4.7: FTIR of ASL

Conc. of protein is 0.3mg/ml

Attempts for crystallization

As the structure of this protein is not known, some **crystallization** attempts were also made and were successful. Shown below are the pictures of crystals of ASL obtained under different conditions.

ASL conc: 13mg/ml in 20mM Tris and 50mM NaCl pH 8

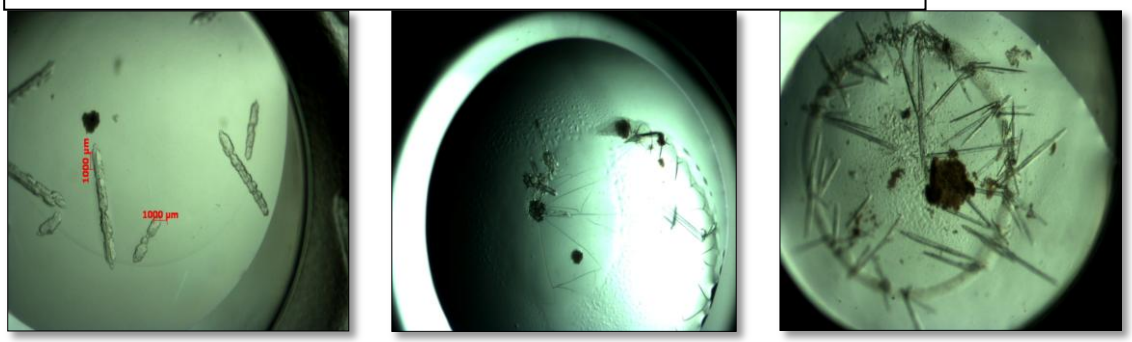


Fig 4.8: Crystal structures of ASL

Salt: 0.2 M MgCl2

Buffer: 0.1M MES pH 6.5

Precipitant: 10% PEG 4000

Buffer: 0.1M tris pH 8.5

Precipitant: 12% PEG 4000

Buffer: 0.1M HEPES pH 7.5

Precipitant: 10% PEG 4000,

5% isopropanol

Differential light scattering (DLS): To find out the hydrodynamic radius of the protein which corresponds to the size of the protein, DLS was done. Figure 17 and 18 represent the DLS profile of ASL.

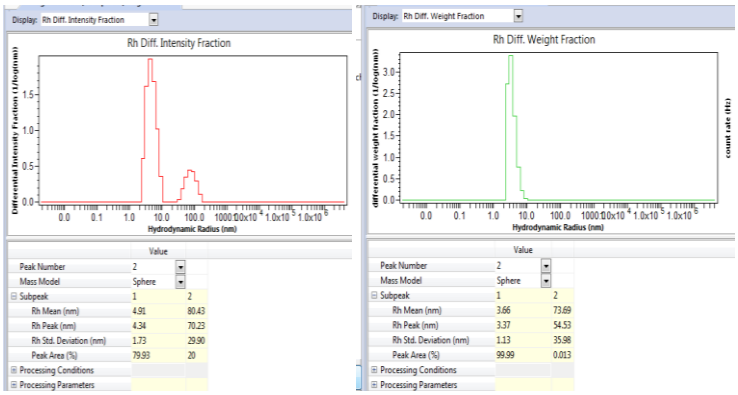


Fig 4.9: Profile of protein which was purified by heating at 70^{o}C for half hour (protein H). Concentration is 3.8mg/ml

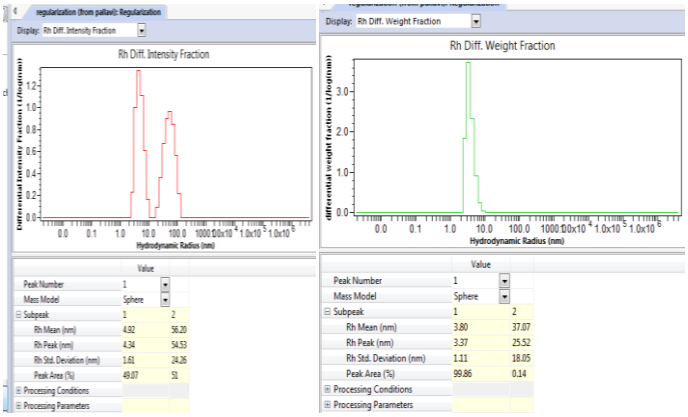
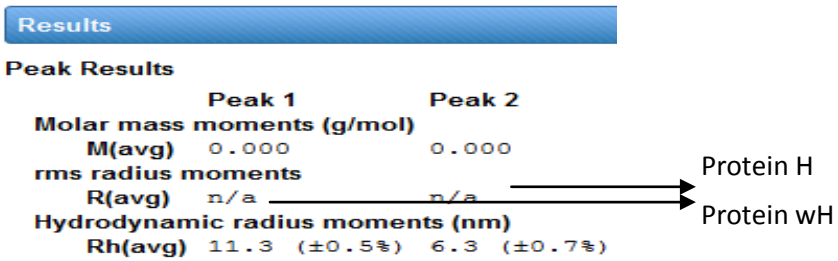


Fig 4.10: Profile of protein which was purified without heating (protein wH). Concentration is 1.9mg/ml



The hydrodynamic radius confirms the protein to be a dimer.

4.1.3 Stability analysis

Thermal melt: The thermal stability of the protein structure (α helix) was checked by subjecting the enzyme to heating from 20 ^{o}C to 90 ^{o}C and cooling down to 20^{o}C

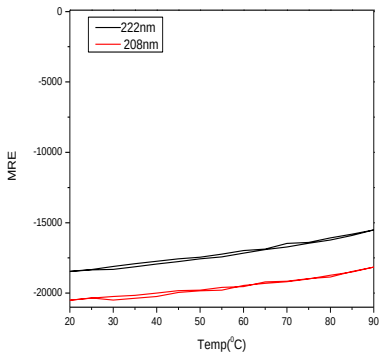


Fig 4.11: Thermal denaturation of ASL (Conc: 0.3 mg/ml)

- Figure 4.11 shows that the protein is highly resistant to temperature as it does not unfold completely and also retraces its path while refolding back to its native structure.

Chemical denaturation: The protein was incubated overnight with the denaturant and boiled for 5 mins at 90^{o}C.

	Stock	**Working**
CTP	1.5mg/ml	0.3mg/ml
Urea	8M	2M, 4M, 6M, 8M
$GuCl_2$	6M	2M, 4M, 6M

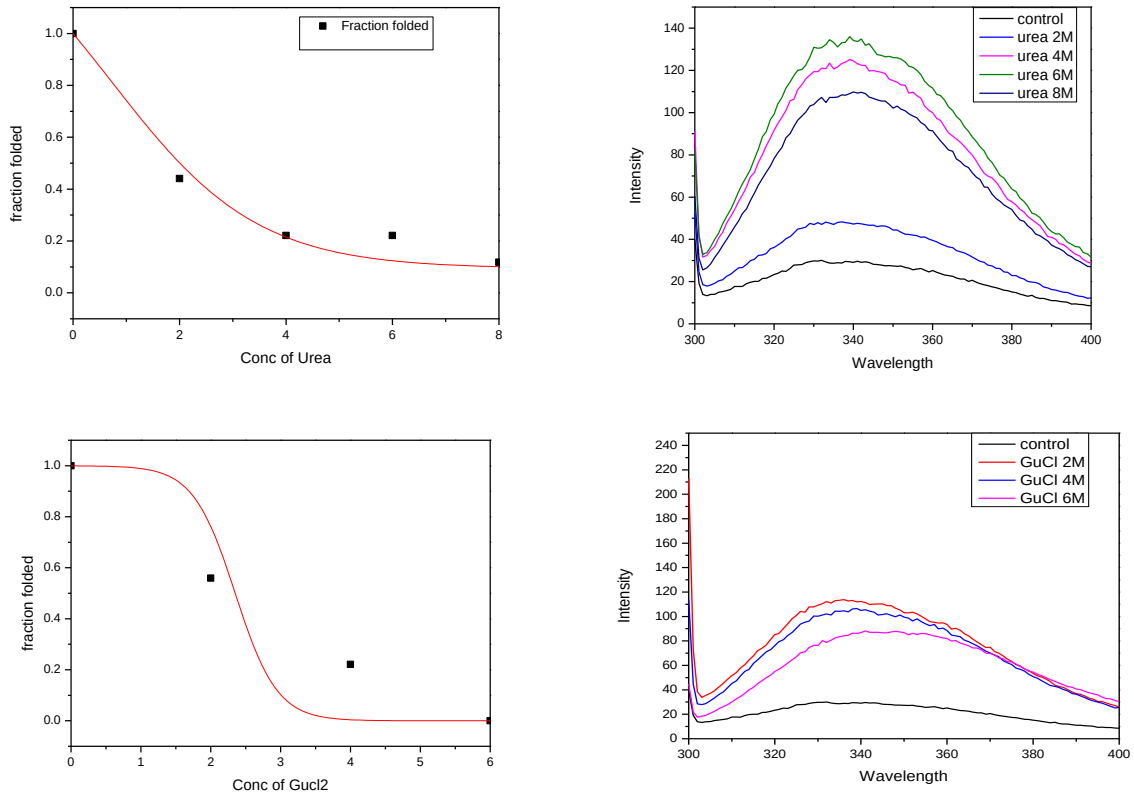


Fig 4.12: Fraction folded versus denaturant concentration and fluorescence emission spectra of ASL at different denaturant concentration.

- The above data suggests that the protein is highly resistant to denaturant as it is not unfolding completely. In case of denaturation by urea the Cm was around 3-4 M while the Cm value was 2.5 in case of guanidine hydrochloride. However, we have to repeat the experiments to finally conclude something.

To analyse the total structure change with chemical denaturation, CD was done on the protein with different concentration of denaturant.

The experiment was done with the protein purified without heating.

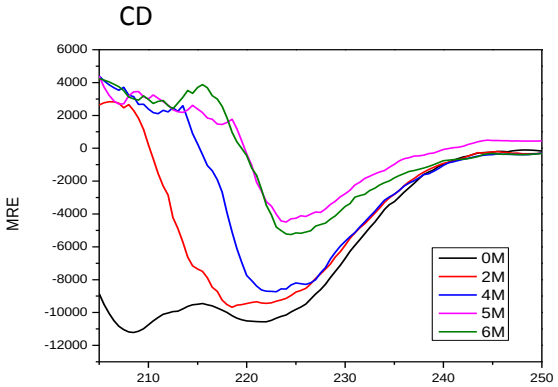


Fig 4.13: CD of ASL during chemical denaturation

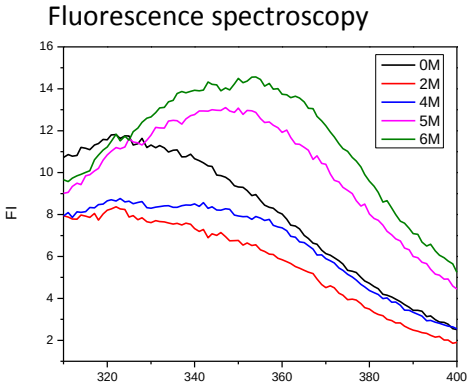
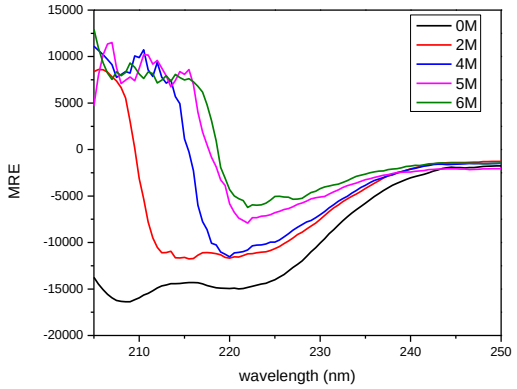


Fig 4.14: Fluorescence Spectroscopy of ASL

Conc: 4.4 µM, Overnight Incubation at 37^{o}C with GuCl: 2M, 4M, 5M, 6M,

The same was done with protein purified by heating at 70 ^{o}C for half hour to check the effect of temperature on structure.



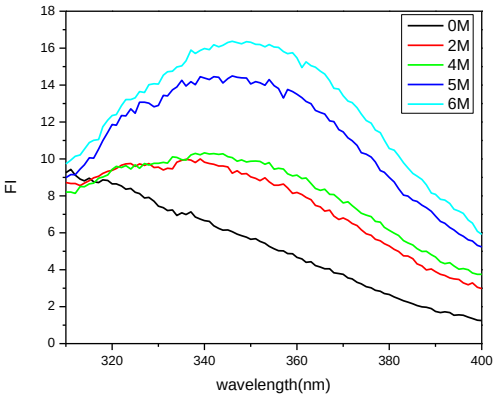


Fig 4.15: CD of ASL (heated) during chemical denaturation

Fig 4.16: Fluorescence Spectroscopy of ASL (heated) during chemical denaturation

unheated and heated protein to see the effect of temperature on the deanaturation profile. The protein structure seems to be disrupted by GuCl but not completely as seen by a dip at 220 nm. The disturbance in CD graph before 215nm is due to increase in HT. Overall, it can be said that the protein is quite resistant to denaturation by GuCl.

Further analysis of protein unfolding profile was done by **Differential Scanning calorimetry (DSC)** to check the cooperative or uncooperative folding of its different domain. The concentration of protein was kept at 0.0089 mM.

The protein was heated from 20^{o}C to 90^{o}C and cooled back to 20^{o}C with a scan rate of 60^{o}C/ hour. For the analysis, the curve was fit and 100 iterations were used until the chi square is not reduced.

DSC

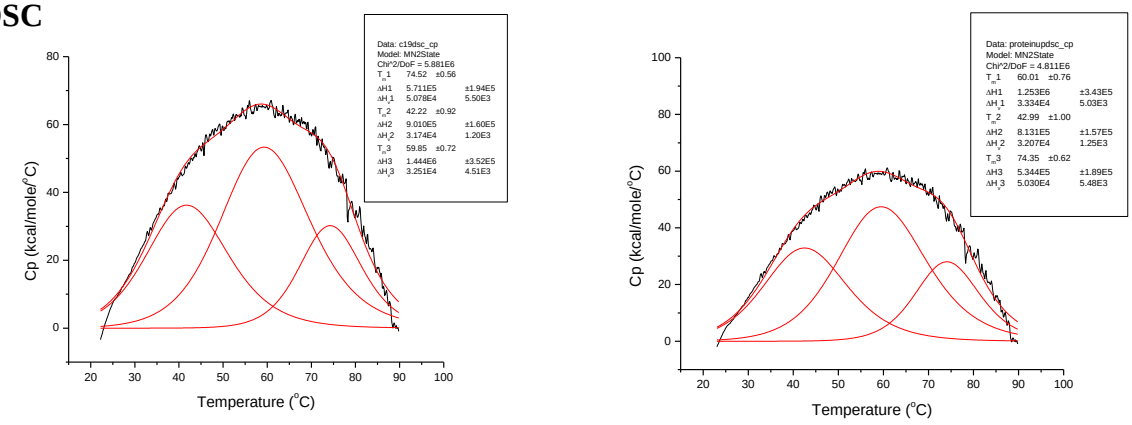


Fig 4.17: Upscan: protein is heated till 90^{o}C

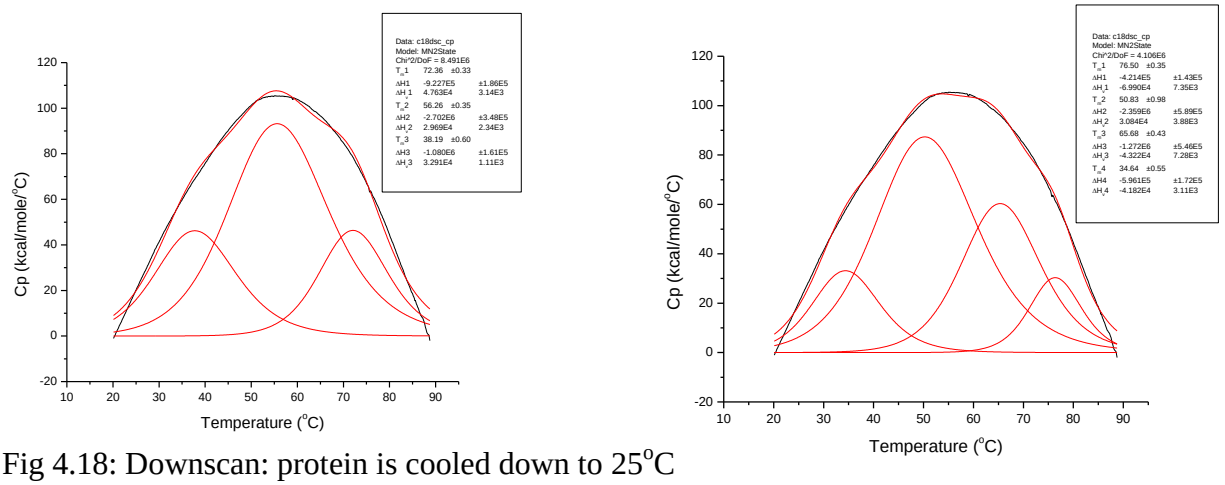


Fig 4.18: Downscan: protein is cooled down to 25^{o}C

- In all the DSC data analysis, it was seen that the protein denatured thermally by 3 to 4 transitions indicating the presence of 3 or 4 domains in the proteins which unfolds at different time points and temperatures in the denaturation experiment. So, it can be concluded from above graphs that all 3-4 domains of protein are unfolding cooperatively.

Mass spectrometry was done to finally confirm the protein sequences.

4.2 Pfu DNA LIGASE

4.2.1 The **amplification** of Pfu Ligase gene was done from genomic DNA by utilizing the following primers.

Primers :
F- ATT AGG ATC CAT GAG GTA TCT AGA GCT TGC
R- ATT ACC CGG GTT AGC TTT CCA CTT TTC C

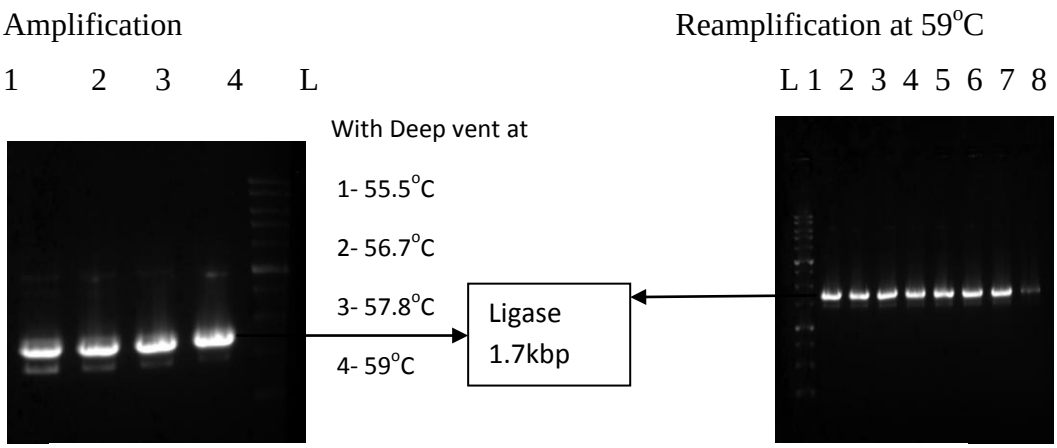
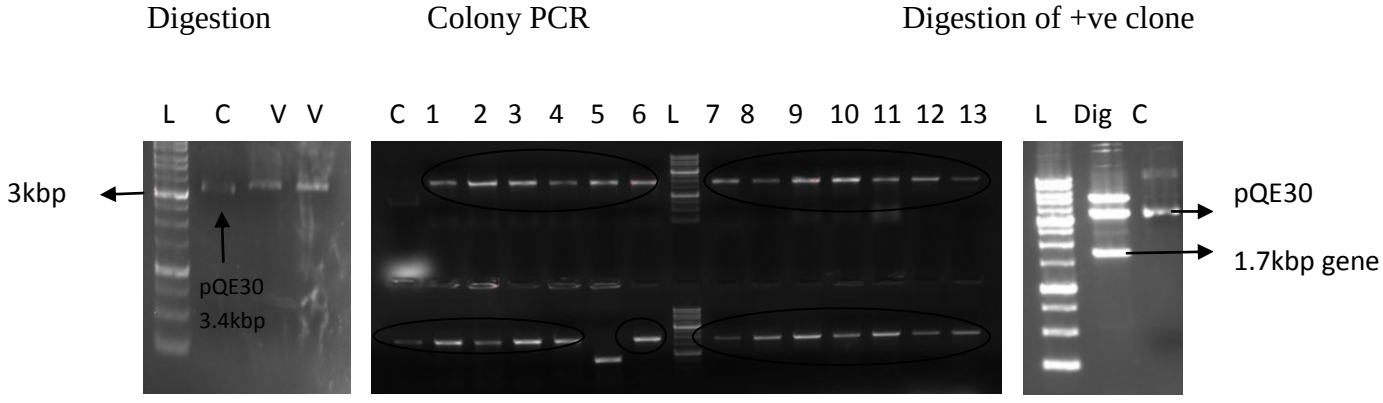


Fig 4.19: PCR of ligase

4.2.2 **Cloning**: Vector (pQE30) and insert (ligase gene) were digested with BamH1 and Xho1 and transformed in XL1B.



L: 1 kb Ladder, C: control, V: digested vector, 1-13: colony numbers, Dig: Cloned vector was digested and check for gene

Fig 4.20: Digestion of Vecto pQE3

Fig 4.21: Colony PCR using vector specific primers

Fig 4.22: Digestion of cloned vector to check for insert

4.2.3 The **expression** of Pfu DNA ligase protein was checked and in in XL1-Blue strain of *E.coli* (Figure 32) and the protein was purified under native conditions by Ni-NTA affinity chromatography.

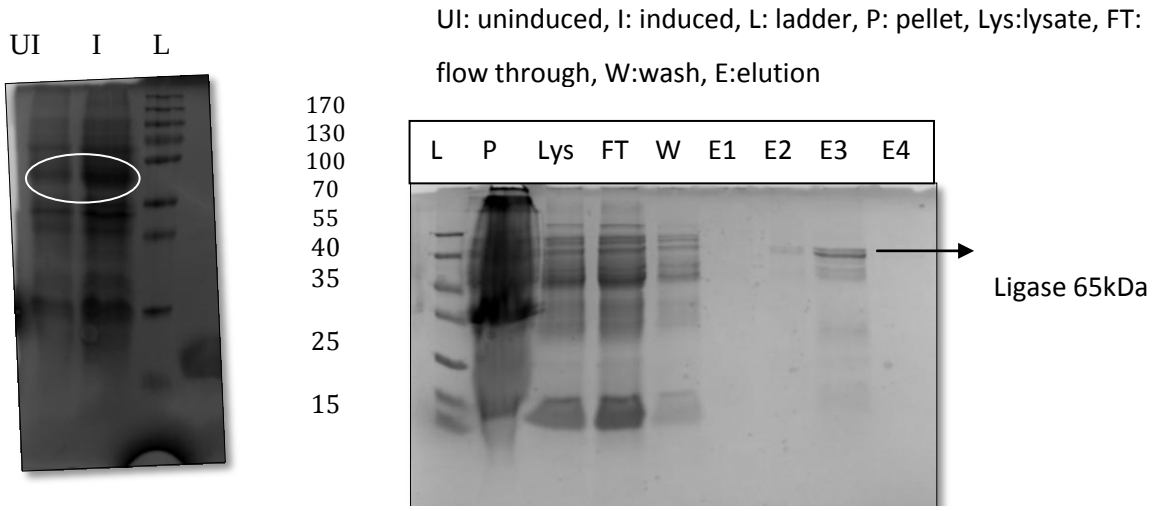


Fig 4.23: Induction and purification of Ligase

The purified protein was prone to degradation and to prevent degradation**,**the protein was heated at 80 ^{o}C for half hour and treated with 1mM PMSF, protease inhibitor cocktail and 1mM EDTA.

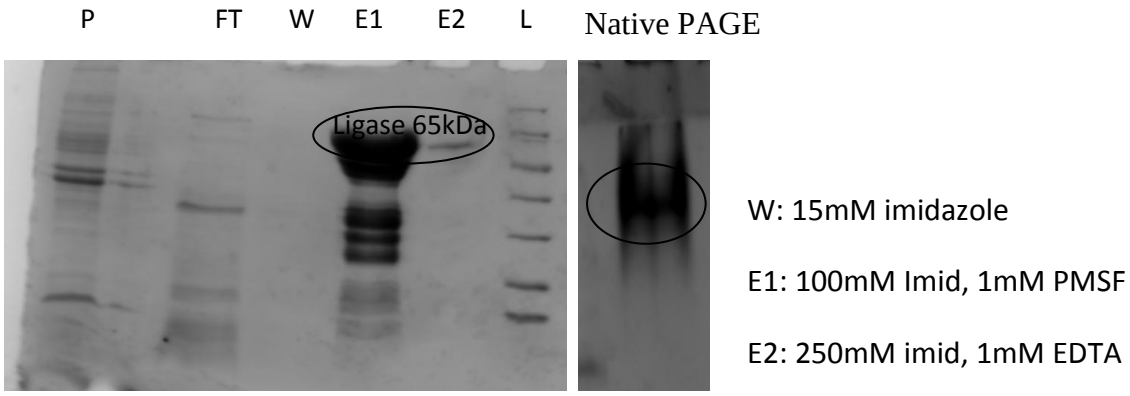


Fig 4.24: SDS and native PAGE of differently treated Ligase protein

- None of the treatments was successful in stopping the degradation but it seems that protein is still associated with each other as seen a single band in Native PAGE (Figure 4.24).

Denaturing purification: To check whether the protein is getting degraded after extracting out of cell or getting degraded inside the cell before lysis, denaturing purification was done.

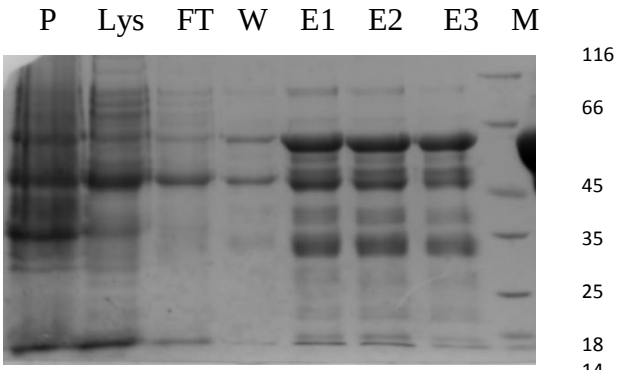


Fig 4.25: Denaturing purification of ligase

- Figure 4.25 shows the presence of multiple bands in the elution fractions showing that the protein is degrading within cell. Also, the band pattern is same in Native and Denaturing purification, further suggesting the degradation of protein within the cell.

4.2.4 **Oligomeric state: FPLC or gel filtration chromatography**

To check the oligomeric state of the protein, gel filtration chromatography was done on Superdex 200 10/300 GL column.The buffer used is 50mM Tris and 100mM NaCl pH 8.

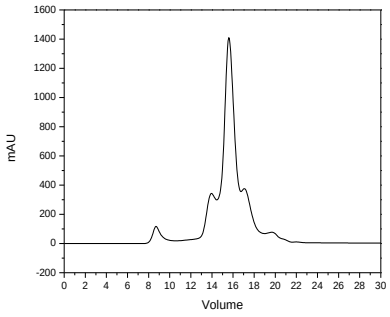


Fig 4.26: Gel filtration of Ligase

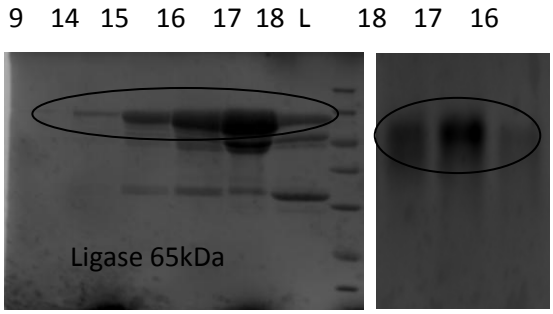


Fig 4.27: Gel filtration elution: SDS PAGE and Native PAGE (Stacking 3% and resolving 6% for Native gel)

FPLC of Protease inhibitor cocktail treated protein

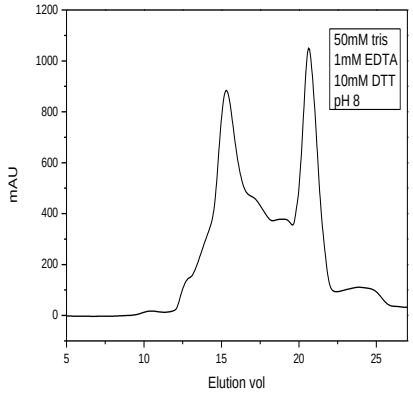
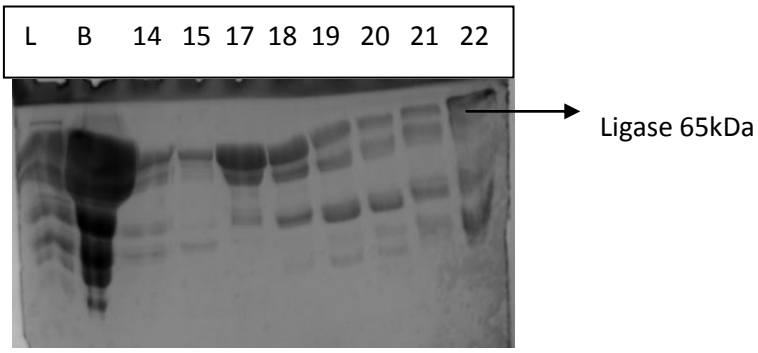


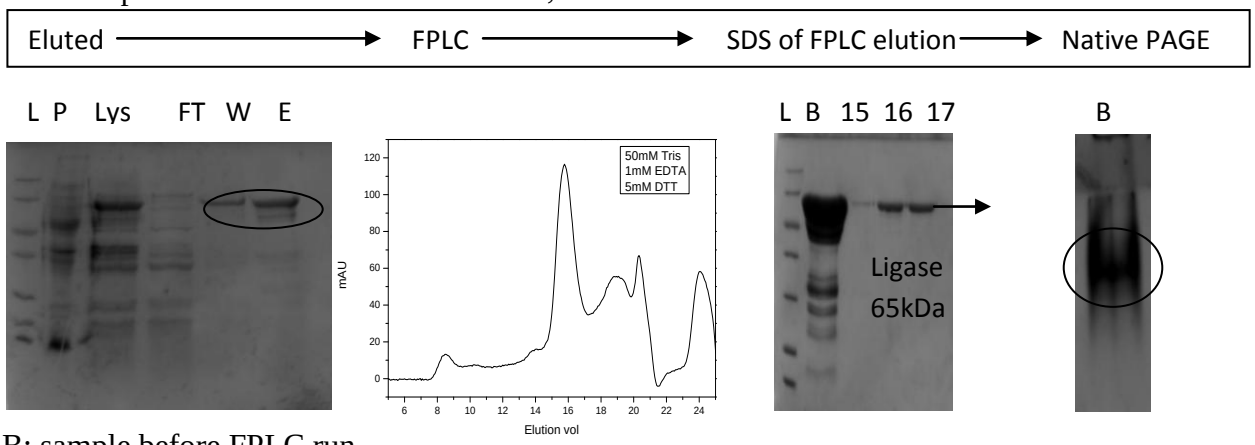
Fig 4.28: Gel filtration of PIC treated ligase



FPLC elution (B: sample before FPLC)

Fig 4.29: SDS PAGE of FPLC eluted

FPLC of protein treated with 2 mM PMSF, 0.5mM EDTA and heated at 80^{o}C for ½ hrs.



B: sample before FPLC run

Fig 4.30: Figure explaining the purification, FPLC, SDS and native PAGE of Ligase

- By comparing with the standard graph, it was inferred that the protein is getting eluted as monomer.

PMF (peptide mass fingerprinting): To confirm whether all the bands are of Ligase, we did PMF.

4.2.5 **Structure analysis**

For all the below analysis, the conc. of protein was kept 0.15mg/ml.

Circular Dichroism: Far UV spectrum for secondary structure analysis

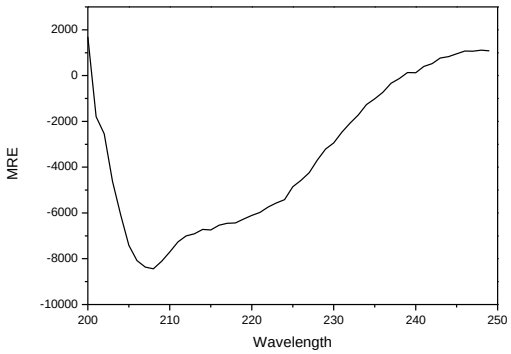


Fig 4.31: CD of Ligase

- It seems that ligase is mostly α helix as seen as a dip at 208nm. Random coil is dominating in the structure.

4.2.6 **Stability analysis**

Chemical denaturation

Fluorescence spectroscopy: The shift in the emission maxima of the intrinsic tryptophan fluorescence of the protein was measured and fraction folded was plotted as a function of denaturant concentration.

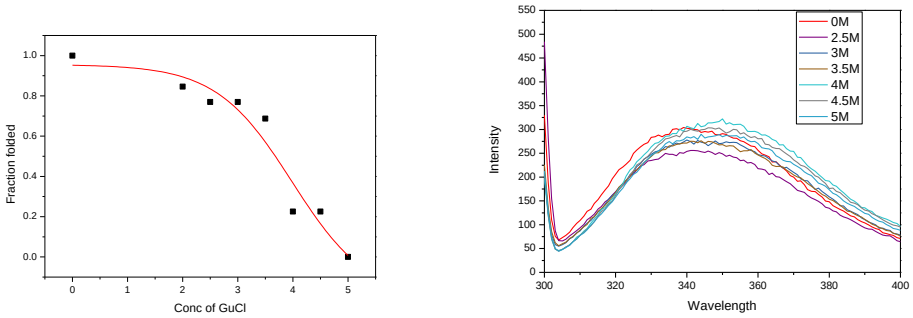


Fig 4.32: Fluorescence spectroscopy of GuCl treated protein

The protein is quite resistant to GuCl denaturation and shows a Cm value of around 3.5M GuCl (Figure 4.32).

Thermal denaturation: The protein was heated to 90^{o}C and cooled back to room temperature.

Thermo-Chemical Denaturation: The protein was incubated with GuCl overnight and folding profile was analysed while heating the protein.

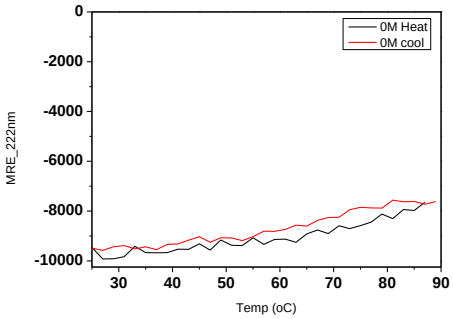


Fig 4.33: Thermal denaturation at 0M GuCl

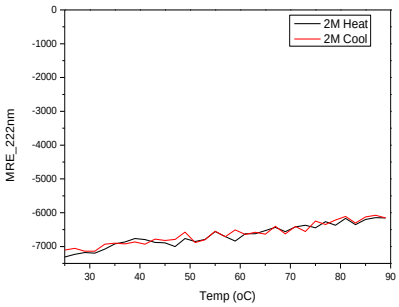


Fig 4.34: Thermal denaturation at 2M GuCl

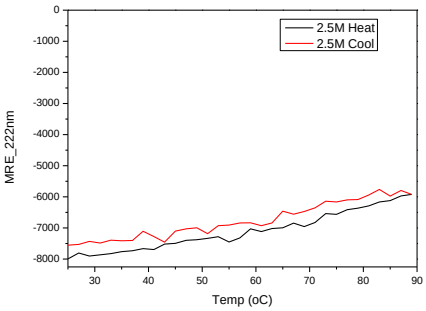


Fig 4.35: Thermal denaturation at 2.5M GuCl

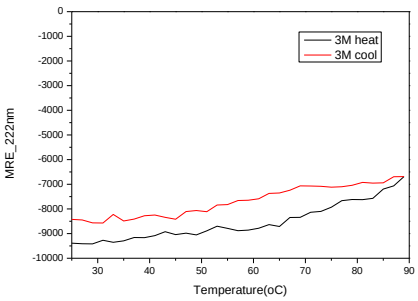


Fig 4.36: Thermal denaturation at 3M GuCl

The above graphs show the protein unfolding and refolding profile with increasing concentration of GuCl and thermal melting. It can be concluded that the protein is quite stable as it does not unfold even after 3M GuCl and 90^{o}C temperature treatment.

The temperature profiling was done to see the effect of temperature on the secondary structure of the protein and to check whether the protein is able to refold after heating to a high temperature.

Structure analysis (**CD**) of protein at all temperature

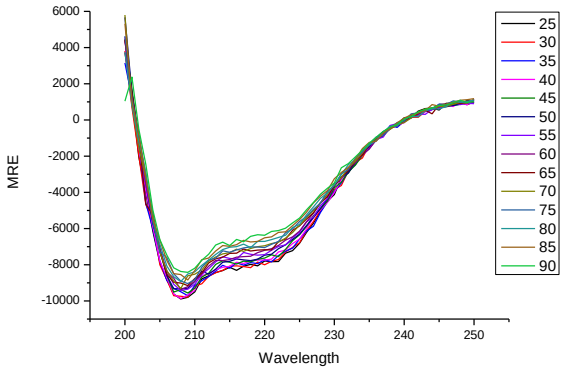
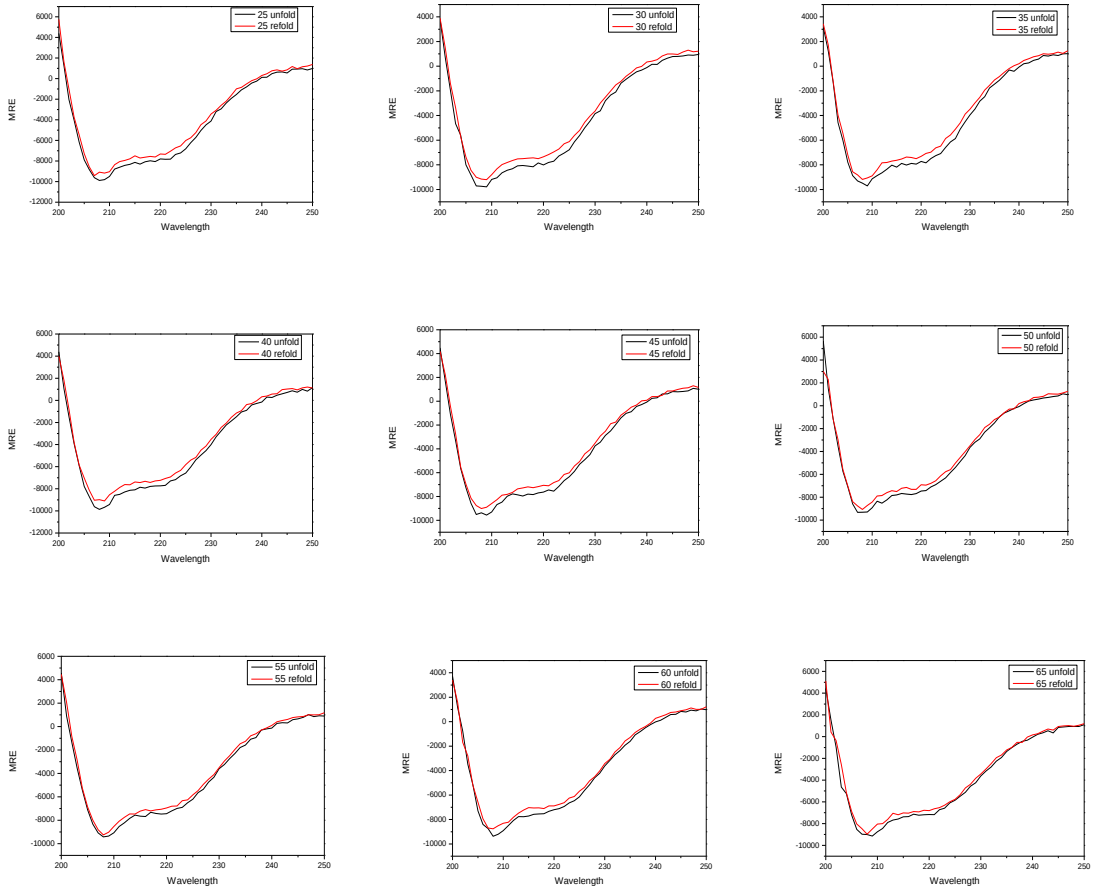


Fig 4.37: CD of Ligase at all temperature

Refolding profiling



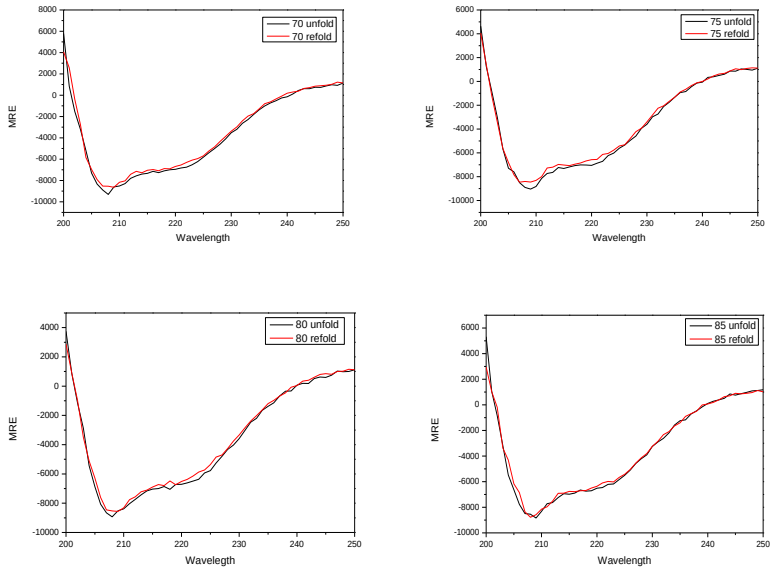


Fig 4.38: The graphs show the unfolding and refolding profile of ligase at all temperatures

The above graphs shows that protein is quite stable, the structure does not disrupt even till 90^{o}C and refold to original position even after heating to 90^{o}C.

4.2.7 **Activity profiling**

Trial 1

pET23a single digested with Hind III → Ligation reaction → PCR

L C 1 L C 1 2 3 4 L 4 1 2 3

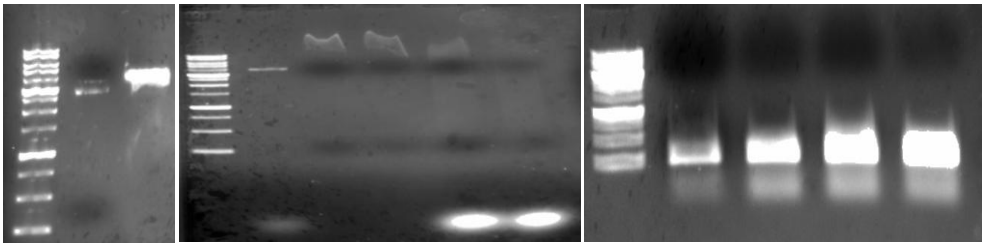


Fig 4.39: Shows the digestion of pET23a, Agarose gel for the ligation reaction and PCR of the ligation mixture

B2: 1:2 dilution of T4lig buffer + 5mM $MgCl_2$

1. B1, 55^{o}C for 45 min
2. B2, 55^{o}C for 45 min

3. B1, 25^{o}C for 3 hrs
4. B2, 25^{o}C for 3 hrs

Pfu ligase was in buffer: 50mM Tris @ pH 7.5

For the activity, 2ul of 1.28mg/ml of pfulig was used in a 20ul reaction. And for the PCR 1ul of the reaction volume was used. The PCR was done at 45 ^{o}C to see whether single digested vector is ligated or not, if it did a band of multiple cloning site (14 kbps) is seen.

- Because of absence of control, can't say whether the band at 14 kbps is of multiple restriction sites (mcs) or not.
- But as this can't be a primer dimer (much intensity), it may be a band of mcs.

Trial 2: The pET23a was singly digested with EcoR1 and purified from agarose gel. This plasmid was tried for the ligation and PCR was done.

L C Dig L 1 2 3 4 5 6

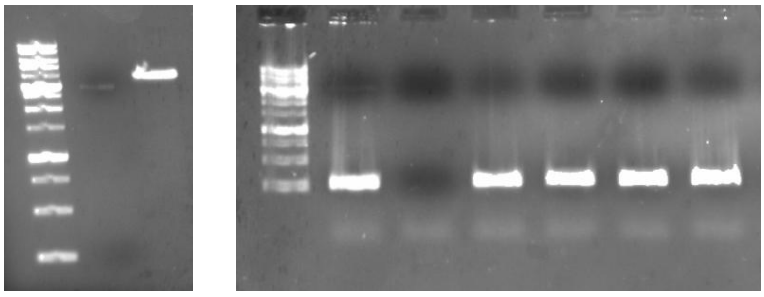


Fig 4.40: Shows the digestion of pET23a and PCR of the ligation mixture

L: Ladder, C: Undigested pET23a, Dig: single digested pET23a

1 and 2 are control; 3-6 are ligation reaction

1ul of 1mg/ml protein was used in 10ul of reaction. For the PCR 2ul of mixture was used.

B1: T4 DNA ligase buffer +10mM NH_4Cl+ 10mM KCl

B2: T4 Ligase buffer was diluted into half+ 5mM $MgCl_2$

3- B1 buffer at 25^{o}C for overnight

4- B2 buffer at 25^{o}C for overnight

5- B1 buffer at 50^{o}C for overnight

6- B2 buffer at 50^{o}C for overnight

1- Undigested control
2- Digested control

- The PCR shows that the single digested product was ligated and multiple cloning sites were amplified in the PCR. As no band is seen in the digested control and a band is seen in undigested control, this can be a positive result. Further analysis has to be done to confirm it.

Trial 3: 11.2ul of 2.6mg/ml of ligase was used in 20 ul of reaction.

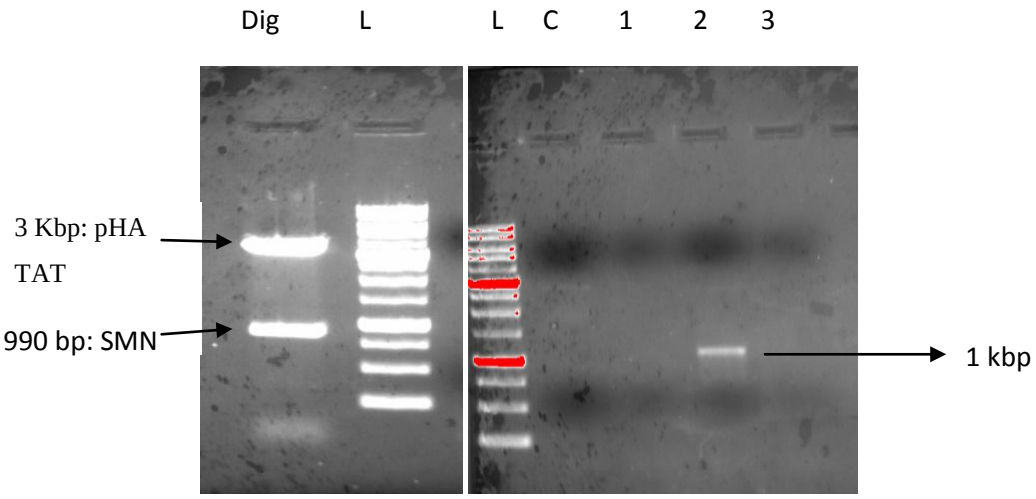


Fig 4.41: Digestion of cloned plasmid and PCR of ligation mixture

C: Cloned plasmid, Dig: Digestion of vector, L: Ladder

1. With pfulig
2. T4 DNA
3. Vector alone

Pfu ligase in 50mM tris and 100mM NaCl at pH8 and Buffer used was T4 DNA Ligase Buffer

- The above result was negative and no amplification was seen in the PCR reaction.

4.3 CARBOXY TERMINAL PROTEASE

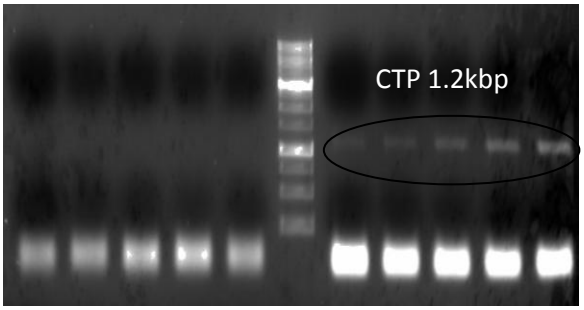
4.3.1 Amplification of the gene was done using the genomic DNA and following primers

Primers:

CTP F: AGCTACTCATATGAGACTCAACCAGATTTCAAAGTTTGC

CTP R: TAACAATGCGGCCGCTTAATGGTGATGGTGATGATGTTTTATTTTCTCCAGTAACACCTC

CTP R (Untag): TTATTTTATTTTCTCCAGTAACACCTC



1-5: tag	6-10: untag
1. 2ng, 50.7°C	6. 2ng, 45.4°C
2. 4ng, 50.7°C	7. 4ng, 45.4°C
3. 6ng, 50.7°C	8. 6ng, 45.4°C
4. 8ng, 52.5°C	9. 8ng, 47°C
5. 10ng, 52.5°C	10.10ng, 47°C

With Deep Vent polymerase

Fig 4.42: PCR for amplification of CTP

Reamplification

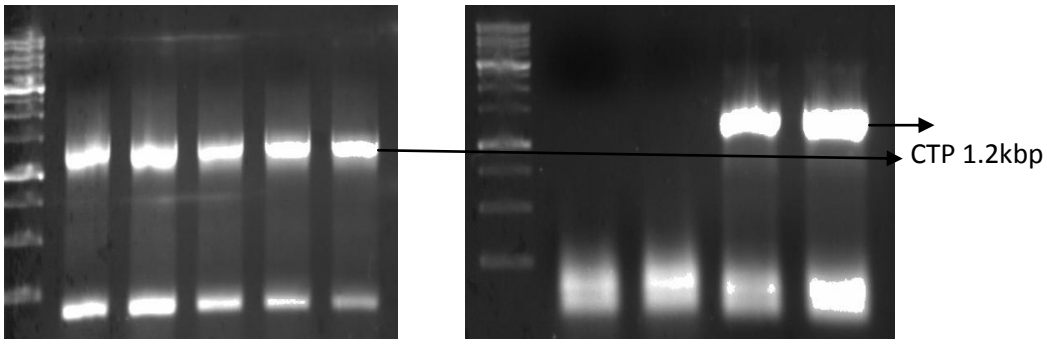
Primer has sequence for 6x His tag at 3'end (tag).

Tagged

L 1 2 3 4 5

1-2: Tag, 3-4: Untagged

L 1 2 3 4



With Deep Vent polymerase

Fig 4.43: PCR for reamplification of CTP

4ng of amplified DNA per reaction was used

0.1 ug of amplified DNA

1. 53.1°C
2. 55°C
3. 59.5°C
4. 61.1°C

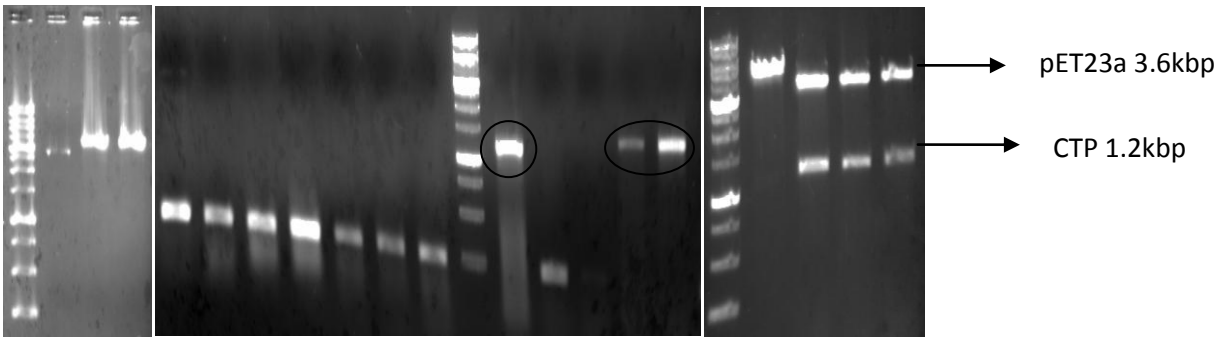
1-2 52.8°C

3-4 46.9°C

4.3.2 Cloning: The pET23a vector and gene was digested with Nde1 and Not1 enzymes and ligated using T4 DNA ligase, and then transformed into XL1B strain of *E.coli*.

Digestion Colony PCR Digestion check of cloned plasmid

L C Dig C 1 2 3 4 5 6 L 7 8 9 10 11 L C 7 10 11



L: ladder, C: undigested Plasmid, Dig: digested pET23a by Nde1 and Not1

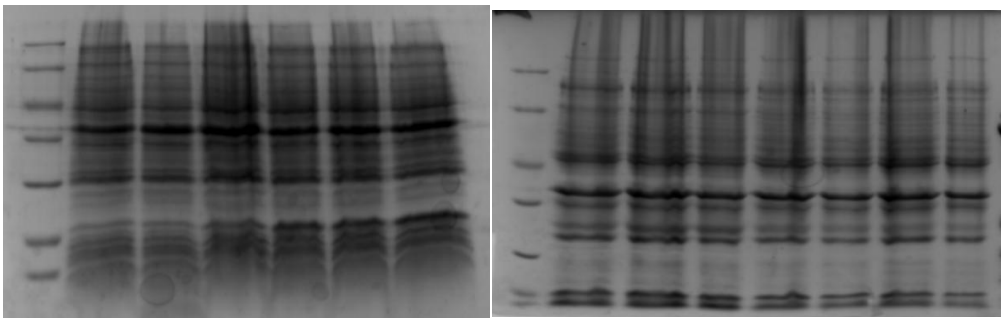
Fig 4.44: Digestion of Vecto pET23a

Fig 4.45: Colony PCR using vector specific primers

Fig 4.46: Digestion of cloned vector to check for insert

4.3.3 Expression: As pET23a is not compatible with XL1B strain of *E.coli,* cloned vector was transformed in BL21 (DE3) and Rossetta strain of *E.coli*.

L UI I I I I I L UI I I I I I I



CTP expression in Rossetta CTP expression in BL21

Fig 4.47: SDS PAGE of CTP induction in *E.coli* strain

- The protein could not be expressed in *E.coli*. Further attempts have to be made to express it in other different expression strain of *E.coli*.

Conclusion

The proteins from Pyroccocus furiosus were successfully cloned and expressed in *E.coli*. Although, both the proteins were getting degraded, still they are intact in native form which has been confirmed by native PAGE as well as Size Exclusion chromatography. Few attempts like PMSF, heating and protease inhibitor cocktail were made to prevent degradation but were unsuccessful. Both the ASL and DNA ligase were very stable chemically as well thermally which was revealed by Circular Dichroism and Fluorescence studies. Further characterization showed that the proteins are mostly α-helical and contain different domains that unfold constitutively. Activity still remained to be explored but few results lead to a path of further exploring the same conditions.

CTP from *Thermotoga maritima* was successfully cloned but protein expression could not be seen in BL21 strain. We changed the expression host to Rosetta DE3 to overcome the problem of rare tRNA in BL21 but still could not see any expression. The reason is still not understood. To further explore the expression profile, different strains of *E.coli*, different ways of transformation, need to be done. After getting expression, activity will be checked.

References

1. C.J. Reed, H. Lewis, E. Trejo, V. Winston, C. evilia : “Protein adaptations in Archaeal Extremophiles”, Archaea, vol 2013, Article ID 373275
2. D.C. Demirjian, F. moris-varas, C.S. Cassidy: “enzymes from extremophiles”, current opinion in chemical biology, 2001, 5:144-151
3. Sapra, R.Bagramyan, K.Adams, M.W.W. (2003): “A simple energy-conserving system: proton reduction coupled to proton translocation”, Proc. Natl. Acas. Sci. U.S.A 100:13, 7545-7550.
4. H. Nishida, D.Tsuchiya, Y.Washino and K.Morikawa: “overexpression, purification and crystallization of an archaeal DNA ligase from *Pyrococcus furiosus*”, Acta Cryst., 2005, F61:1100-1102
5. Camilla L., Neasbo, Stephane I Haridon, Karl O.Stetter & W. Ford Doolittle: “Phylogenetic analyses of two “archaeal” genes in Thermotoga maritima reveal multiple transfers between Archaea and Bacteria”, Mol. Bio. And Evol. 18 (3): 362-375
6. MEROPS: peptidase database
7. Y. Yamamoto, N. Inagaki, K. Satoh: “Overexpression and characterization of carboxyl-terminal processing protease for precursor D1 Protein”, J Biol Chem., 2001, Vol 276, pp 7518-7525
8. Anthony Day: “the source of stability of proteins”, 1996
9. Vielle C. and Zeikus J.: “Thermozymes: identifying molecular determinants of protein structural and functional stability”, Trends in Biotechnology 14, 183-90 (1996)
10. S. C. S. Nagamani MD, A. Erez MD, PhD & B. Lee MD,PhD: “Argininosuccinate Lyase deficiency”, Genetics in medicine, 2012, 14 :501-507
11. Sellek GA, Chaudhuri JB: “Biocatalysis in organic media using enzymes from extremophiles”, Enzyme Microb Technol 1999, 25:471-482
12. H. Nishida, S. Kiyonary, Y. Ishino & K. Morikawa: “The closed structure of an Archaeal DNA Ligase from *Pyrococcus furiosus*”, J. Mol. Biol., 2006, 360: 956-967
13. Ramachandran G.N., C. Ramakrishnan, V. Sasisekharan: “Stereochemistry of polypeptide chain configurations”, J Mol. Biol., 1963, 7:95-9
14. “Folding Funnel”, Wikipedia