Recombinant expression of YchP outer membrane protein from *Salmonella enterica serovar* Typhimurium and its biophysical characterization

M.Sc. Thesis

By
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BIOSCIENCE AND BIOMEDICAL ENGINEERING

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A THESIS

Submitted in partial fulfillment of the
requirements for the award of the degree
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Sushma Ahirwar

BIOSCIENCES AND BIOMEDICAL ENGINEERING

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"Recombinant expression of YchP outer membrane protein from *Salmonella enterica serovar* Typhimurium and its biophysical characterization"** in the partial fulfilment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING, Indian Institute of Technology Indore,** is an authentic record of my own work carried out during the time period from June 2022 to May 2023 under the supervision of Dr. Prashant Kodgire Professor, Department of Biosciences and Biomedical Engineering.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

Sushma 9/5/23

Signature of the student with date
(Sushma Ahirwar)

This is to certify that the above statement made by the candidate is correct to the best of my/our knowledge.

P.V. Kodgire

Signature of the Supervisor of M.Sc. thesis

(Dr. Prashant Kodgire)

Ms. Sushma Ahirwar has successfully given his/her M.Sc. Oral Examination held on **8th May 2023.**

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Sushma Ahirwar

DEDICATED TO

My Beloved Family and Friends

Abstract

Salmonella enterica serovar Typhimurium is a gram-negative pathogenic bacterium. OMPs from gram-negative bacteria play various functional roles including causing virulence. Till now few of OMPs have been studied in *Salmonella* such as OmpC, OmpD, OmpA, OmpF, etc. YchP is another putative invasin OMP from *Salmonella*, which has an affinity for β-integrin present on the mammalian host cell surface and plays an important role in invasion into host cells and causing virulence. In-silico analysis of YchP (~52kDa) shows that it is a β-barrel OMP, containing 41 residue long signal peptide and 14 tryptophan residues. The biophysical study of YchP indicate that it may be exists as a monomer. Refolding and secondary structure analysis of YchP were done under different conditions of buffer containing urea (denaturant), lipids, and detergents using techniques such as tryptophan fluorimetry, CD spectroscopy, and ATR-FTIR spectroscopy. Our study provides the structural information of YchP OMP, which may help us in understanding the pathogenesis caused by YchP OMP in *Salmonella*.

Keywords: Gram-negative bacteria, OMP, invasin, integrin, pathogenesis.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Abbreviations

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

OMP- Outer membrane protein

PCR - Polymerase chain reaction

r-YchP- Recombinant YchP

SDS PAGE- Sodium dodecyl polyacrylamide gel electrophoresis

CD-spectroscopy- Circular dichroism spectroscopy

SEC- Size exclusion chromatography

DDAO- N, N-Dimethyl-1-Dodecanamine-N-Oxide

DOPC- 1,2-dioleoyl-sn-glycero-3-phosphocholine

DMPC- 1,2-dimyristoyl- 5n -glycero-3-phosphocholine

CMC- Critical micelle concentration

ATR-FTIR- Attenuated total reflectance -Fourier transform infrared (FTIR) spectroscopy

Chapter 1

Introduction

Gram-negative bacteria are surrounded by a two-phospholipid bilayer membrane-inner cytoplasmic membrane and outer membrane. Peptidoglycan layer sandwiched between inner and outer membrane. There is periplasmic space present between the outer membrane and the peptidoglycan layer. The outer membrane in gram-negative bacteria is highly asymmetric and proteins present in the outer membrane region provide unique and distinct features to gram-negative bacteria. 50% OMPs (outer membrane proteins) are beta stranded forming a barrel shape (porin). OMPs are playing various important roles in gram-negative bacteria such as the transport of solutes and nutrients (OmpF, OmpA, etc.) across the bacterial cell membrane, acting as a physical barrier, protecting bacteria from harsh conditions such as an antimicrobial attack, signal transduction, host-pathogen interaction (TonA, a receptor for phage λ) [4]; involve in cell-to-cell communication system [4]; or quorum sensing (OprF) [9], etc. Because of all such roles, scientists are interested in studying the structure and function relationship of these various OMPs. These OMPs can be monomeric, dimeric, trimeric, or oligomeric based on their structure. OMPs are synthesized in ribosomes present in the cytoplasm of gram-negative bacteria. Nascent polypeptide chain containing signal peptides are transported across the membrane from the inner membrane to periplasmic space via Sec translocase and the BAM assembly complex recognizes the signal peptide containing nascent OMP and integrate them into the outer membrane of gram-negative bacteria. Misfolded OMPs are degraded by proteases.

According to WHO 550 million people fall ill each year due to diarrhoeal diseases caused by intake of unhygienic food and water, which include 220 million children under the age of 5 years. *Salmonella* is one of the key global causes of diarrhoeal diseases [3].

Salmonella is a gram-negative, flagellated, facultative anaerobic, and rod-shaped bacterium, belonging to the Enterobacteriaceae family. There are two majorly known species of *Salmonella* – *Salmonella bongori* and *Salmonella enterica*. Over 2,500 serotypes of *Salmonella enterica* have been identified till now, in which *Salmonella enterica serovar* Typhimurium is a non-typhoidal serotype causing diarrhoeal diseases such as gastroenteritis. Non-typhoidal *Salmonella enterica serovar* Typhimurium infection is endemic to southeast Asian countries including Malaysia, Malawi, Singapore, India etc., and sub-Saharan Africa [1,2].

Till now various OMPs have been studied in *Salmonella* which are involved in pathogenesis and virulence caused by *Salmonella*, few of those OMPs are as follows- OmpC, OmpD, OmpA, OmpF [8]; etc. **YchP** is another OMP from *Salmonella enterica serovar* typhimurium which belongs to the putative invasion family of OMPs and its size is around 52kDa. These invasin family OMPs are involved in the adhesion or entry of the gram-negative pathogen into host mammalian cells via having a strong affinity to β-integrin present on the mammalian host cell surface. Invasin family OMPs shows high affinity to β-integrin such as $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_3\beta_1$ [7].

Till now there is no significant study has been done on the biophysical perspectives of YchP OMP from *Salmonella*. So here in this study we have performed a biophysical characterization study of YchP, putative invasin OMP from *Salmonella enterica serovar* Typhimurium, which involve in virulence caused by *Salmonella*.

Chapter 2

Project Overview and objectives

YchP is a putative invasin OMP *from Salmonella enterica serovar* Typhimurium. In this project, we performed a cloning, expression of r-YchP OMP from *Salmonella enterica serovar* Typhimurium strain into *E. coli* expression host. Then we performed solubilization, purification, and refolding of expressed r-YchP OMP and proceeded to a biophysical study of purified, refolded r-YchP OMP that include oligomerization or dimerization study via size exclusion chromatography and secondary structure analysis of expressed r-YchP OMP using various techniques such as CD spectroscopy, tryptophan fluorimetry under different conditions of denaturant, detergents, and lipids and performed heat modifiability assay, ATR-FTIR for refolded beta barrel YchP OMP, etc. The purpose of doing biophysical characterization is to get a better understanding of the structure and functional relationship of YchP OMP.

Objectives-

1. Cloning, expression, and purification of r-YchP OMP from *Salmonella enterica serovar* Typhimurium strain 14028s into pET43a.

2. Biophysical characterization of expressed and purified r-YchP outer membrane protein.

Chapter 3

Material and methods

3.1 Materials

3.1.1 Strains

Salmonella enterica serovar Typhimurium 14028s strain, *E. coli* cell lines, DH5α and Rosetta, were used as host cells for cloning and the pET43a vector (Novagen) was used for cloning and expressing the protein.

3.1.2 Chemical Reagents and Kits

Agarose special (HiMedia), Luria Bertani broth (HiMedia), Luria Bertani agar (HiMedia), Ampicillin (HiMedia), Restriction endonucleases – *Nde*I, *Xho*I, *Sca*I, *Hinc*II, *Ssp*I (NEB), *Taq* DNA polymerase, *Pfu* DNA polymerase, T4 DNA ligase, DNTPs, Taq Buffer, HiProof Buffer, Ethidium bromide (Sigma Aldrich), Favor Prep plasmid extraction mini kit, GeneJET gel extraction kit (Thermo Scientific), GeneJET purification kit (Thermo Scientific), Tris-Cl, NaCl, Imidazole (Sigma Aldrich), $CaCl_2$, Bradford (HiMedia), PMSF (HiMedia), LDAO (SRL), Biorad resin or Ni-NTA beads, Tween 20 (SIGMA-ALDRICH), Sodium hydrogen phosphate (Na_2HPO_4), Sodium dihydrogen phosphate (NaH_2PO_4), Ethidium bromide (EtBr), Sodium chloride (NaCl), Sodium dodecyl sulphate or Sodium lauryl sulphate (SDS), Commassie brilliant blue G-250, β-mercaptoethanol ($HOCH_2CH_2SH$), Agarose, Luria-Bertani agar (LB agar), Luria-Bertani broth (LB broth), Ampicillin, Kanamycin, Acrylamide (C_3H_5NO), N,N′-Methylene bis-acrylamide (MBAA), Triethylenetetramine or Tetramethyl ethylene diamine (TEMED), Ammonium per sulphate ($(NH_4)_2S_2O_8$), Glycerol ($C_3H_8O_3$), Methanol (CH_3OH), DOPC (SRL), DMPC (SRL).

3.2 Experimental methods

Cloning strategy for cloning and expression of YchP outer membrane protein from *Salmonella enterica serovar* Typhimurium strain 14028s into pET43a vector at *Nde*I and *Xho*I site**.**

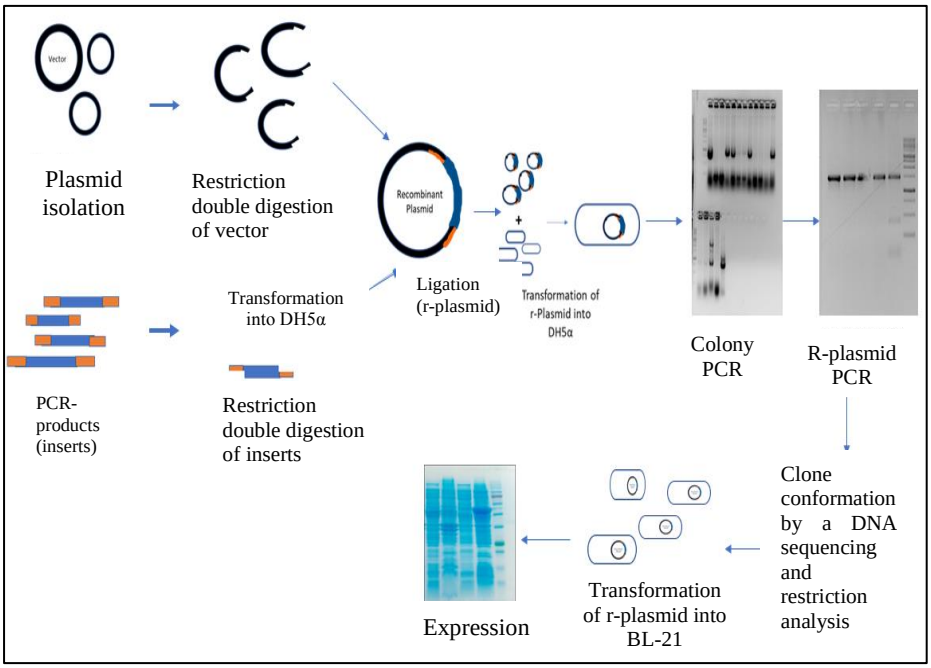


Fig. 3.1 Schematic diagram for cloning strategy

3.2.1 Plasmid Isolation

E. coli cell containing the required plasmid pET43a was grown overnight for at least 16 hours in Luria Bertani broth containing ampicillin antibiotic. The plasmid was isolated from *E. coli* cells using a plasmid extraction miniprep kit. The well-grown bacterial culture was pelleted down at 14000x g centrifugation speed for 10 minutes. Then the pellet was resuspended by pipetting with FAPD1 buffer which contains RNase A. Then FAPD2 (lysis buffer) buffer was added to the resuspended cells and mixed with the cells by gently inverting the tubes 5-10 times and incubated for 2-3 minutes at room temperature 5 minutes. Then FAPD3 buffer (Neutralizing buffer) was added to the lysed cells and gently inverted the tubes 5-10 times immediately. Then the tubes were centrifuged at full speed (18000x g) to clear the lysate. Then the supernatant was transferred into the FAPD column and

centrifuged again at 11000x g for 30 seconds. The flow-through was removed, followed by the addition of W1buffer to the FAPD column and the column was again centrifuged at 11,000x g for 30 seconds. The flow-through obtained is thrown away and wash buffer (EtOH added) is added to the FAPD column, followed by centrifugation for 30 seconds at 11,000x g. The flowthrough was discarded and centrifuged the column at high speed for an additional 3 min to dry the column. Then the FAPD column was placed into a fresh microcentrifuge tube and the plasmid was eluted by adding the 50 µl of elution buffer at the Centre of the membrane of the column and centrifuged at high speed for 2 minutes.

3.2.2 PCR (polymerase chain reaction)

The inserts were prepared via PCR amplification of genomic DNA using the forward and reverse primer. The master mixture was prepared to contain the essential reagents including- forward primer, reverse primer, genomic DNA template, DNTPs, Hi-Proof buffer, Pfu DNA polymerase, and autoclaved distilled water to cover up the remaining volume. Then the master mixture was aliquoted into PCR tubes. The PCR tubes containing the master mixture were put into the thermocycler and set the PCR program in the thermocycler as the initial denaturation temperature was 95 ˚C for 4 minutes, followed by a denaturation temperature of 95 ˚C for 30 seconds, and from this temperature, program was set up to 30 cycles, annealing temperature according to the primers Tm for 30 seconds, extension temperature was set at 72˚C, extension time depending on the length of the template, (here for *ychP* annealing was done in 55-60˚C range) and polymerization rate of polymerase enzyme and the final extension was 72˚C for 10 min. and storage temperature was at 4˚C for infinite time.

3.2.3 Restriction double digestion

pET43a plasmid was digested sequentially using restriction endonucleases. The reaction mixture contained the components as follows- cut-smart buffer, template (plasmid/insert), and restriction endonucleases. First, the plasmid was digested using single restriction

endonuclease (here *Nde*I was used first), and incubated at 37˚C for 2-3 hrs. After complete single digestion 2nd restriction endonuclease (here *Xho*I used) was added to the reaction mixture and incubated at 37˚C for 2-3 hrs. After complete double digestion, the enzymes were heat inactivated at 65˚C for 20 min.

3.2.4 Agarose gel electrophoresis

DNA and RNA can be separated on an agarose matrix under the effect of the applied electric field. Separation is based on the difference in the migration of molecules based on their size, shape, and charge. Larger will migrate slowly and smaller fragments will run fast. The agarose powder was poured into 1x TAE buffer and melted at 340 W for 2-3 minutes and EtBr added melted agarose was poured into gel caster unit and allowed to solidify properly for 30 minutes and well formed by immediately inserting the comb into poured melted agarose. The gel electrophoresis unit was filled enough with 1x TAE buffer so that the solidified agarose was immersed enough into the TAE buffer. Here EtBr help in visualizing the DNA fragments after UV illumination. The prepared DNA sample (plasmid/ insert) is loaded into wells after mixing with DNA loading dye containing glycerol which provides density so that sample will not bump out from the wells and bromophenol blue will help us trace the movements. The wells were loaded with samples and an electric field was provided. Then after enough migration (1/4th of gel caster) the separated DNA fragments were visualized under a UV illuminator.

3.2.5 Gel purification of digested DNA fragments (vector and inserts)

The digested DNA fragments were extracted from the gel using the GeneJET purification column. The agarose gel having the DNA fragments was cut into pieces using a clean scalpel and the pieces were placed into the fresh-weighed microcentrifuge tubes, and the weight of the gel slice was recorded. An equal volume of the binding buffer is added to the gel. To completely dissolve the gel, gels were incubated at 50°C -60°C, and the tubes were inverted and mixed until the gel was

completely dissolved. The gel solution was transferred to the column and centrifuged for 1 min. The flow-through so obtained was thrown away and the column was washed with the wash buffer by centrifuging them for 1 minute. To remove residual wash buffer column was spun for additional 3 minutes at high speed. The DNA was obtained after eluting with 50µl of elution buffer and stored at 4° C.

3.2.6 Ligation

Restriction double-digested DNA (vector and insert) with restriction enzymes was ligated using T4 DNA Ligase and 1x ligation buffer. The reaction mixture was incubated at 22° C for 2 hours. The ligation was performed in an I: V=3:1 ratio and the overall DNA amount keep constant to 300ng.

3.2.7 Transformation

The transformation was done by the heat shock method. Competent cells thawed on ice for some time. 5 µl of ligated DNA mixture was taken into the microcentrifuge tube containing thawed DH5α competent cells and incubated for 30 minutes on ice and then gave heat shock by incubating tubes at 42° C for 90 seconds followed by 2 minutes of incubation on ice. 4 times of competent cell, LB broth was added to each tube and incubated for 1 hour 30 minutes at 37° C. Transformed cells were then spread onto the LB agar antibiotic plate.

3.2.8 Colony PCR

Colony PCR was performed to analyse the ligation of the insert into the vector. A colony of bacteria was initially replica plated on a fresh plate and then added as the template instead of the DNA to the master mix of the PCR following the same procedure as described for the PCR.

3.2.9 Recombinant plasmid PCR

Recombinant plasmid PCR was performed to analyse the ligation of the insert into the vector. An isolated recombinant plasmid (r-plasmid) was isolated from the positive result colony PCR result showing bacterial colonies and then added as the template instead of the DNA to the master mix of the PCR following the same procedure as described for the PCR.

3.2.10 Restriction analysis of recombinant plasmid

The conformation of r-plasmid was also done by restriction analysis. In restriction analysis, r-plasmid was used as a template, and used the restriction endonuclease for the site at which the desired gene was cloned and provided appropriate conditions such as cut-smart buffer, Optimum working pH for enzymes, and incubated the reaction mixture at a suitable temperature or time as mentioned for the proper working of enzymes. Then the digested products were analysed on 1% TAE agarose gel.

3.2.11 Recombinant protein expression

Resultant recombinant plasmids were transformed into *E. coli* Rosetta (DE3) cells and induced with isopropyl-β-d-1-thiogalactopyranoside (IPTG). Induced cells were harvested, washed, and resuspended in 1mL of buffer containing 20mM Tris-Cl, pH 7.6, 200mM NaCl, and 10% glycerol. Cell lysates were prepared by sonication at 65% amplitude for at least 2 minutes. and separated as supernatant and pellet by centrifuging it at 11,000 rpm for 10 minutes at 4°C and supernatant and pellet samples were analyzed on SDS-PAGE gels to check the expression of outer membrane protein.

3.2.12 SDS PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) is a technique to separate the protein onto the gel depending on their size by applying the electric field and discontinuous polyacrylamide gel as the support medium. SDS in the gel imparts a negative charge thus covering the charge of the protein depending on the charge-to-mass ratio. In the discontinuous SDS PAGE, first, the protein migrates into the stacking gel of pH 6.8 and then through the resolving gel of pH 8.8. The difference in pH leads to the stacking effect at the junction between the stacking gel and the separating gel. The running buffer mainly contains Tris-glycine with SDS. When the external electric field is applied protein migrates towards the positive electrode anode with a different migrating speed. The smaller protein travels a larger distance compares to the larger protein. At the end of the SDS PAGE electrophoresis, the gel is stained in the Coomassie dye. The

amount of protein can also be estimated using the gel by the intensity of the band. Samples were prepared using 1X loading dye and heated at 95° C for 5 minutes. Samples were loaded and the gel was run in the 1X running buffer. The gel was stained and destined for the required period and the gel was analysed.

3.2.13 r-YchP OMP expression, solubilization, and purification

E. coli (BL-21) colony carrying r-YchP pET43a plasmid was inoculated into prepared autoclaved LB broth with 100µg/mL ampicillin and incubated for 16 hrs at 37°C, 220 rpm for primary culture. Then cells were secondary cultured in 800ml fresh autoclaved LB media and incubated for 3 hrs at 37°C,220 rpm (until OD reached 0.6). Then cells were induced with 1mM IPTG followed by incubation for 4 hrs. at 37°C, 220 rpm. After completion of induction cells were pelleted down at 10,000 rpm for 10min. The pelleted cells were washed with tris buffer pH 8.0 (50mM tris-Cl, 500mM NaCl) and again pelleted down the washed cells at 10,000 rpm for 10min. The washed cells were resuspended in tris buffer (50mM tris-Cl, 500mM NaCl, 6.8µg or 0.0068mg PMSF and proceeded for cell lysis by sonication for 30min. at pulse 2 on and 2 off and 65% amplitude. Then the lysed cells were centrifuged at 11,000rpm for 30min. so the supernatant got separated and cell debris was pelleted down. Induced was checked by running a sample on 12% SDS PAGE gel, the induced protein present in the inclusion bodies or pellet, so the pellet proceeded for the solubilization. For Solubilization induced pellet was washed with buffer containing 50mM tris (pH 7.4), 500mM NaCl, and 2M urea and centrifuged for 10min at 10,000rpm. Then the pellet was resuspended with 20ml solubilization buffer of pH 7.5 containing 50mM tris-Cl pH 6.7, 500mM NaCl, and 8M urea, 20mM imidazole and incubated at room temperature for 2-4 hrs. and then centrifuged at 12,000 rpm at 20°C for 30-40 min. so the supernatant got separated and the insoluble part was pelleted down. And checked the solubilization by running the supernatant sample and pellet after solubilization on 12% SDS PAGE gel. The solubilized supernatant proceeded for purification. Purification

was done by gravity column. 1ml of Bio-Rad resin or beads were introduced into the column and washed with 2-column volume autoclaved distilled water and then charged with 5 ml of 0.1M $NiSO_4$.Charged beads were again washed with 1 column volume autoclaved distilled water and then equilibrated with equilibration buffer pH 7.5 containing 50mM tris-Cl pH 6.7, 500mM NaCl, and 8M urea, 20mM imidazole. Then the solubilized supernatant sample was incubated with beads for 1hr and then passed through the gravity column and flowthrough was collected in a fresh falcon tube. Then washes were provided with tris buffer containing 50mM Tris pH 8, 500mM NaCl, and imidazole with varying concentrations. For YchP outer membrane protein three washes were provided (100ml W1 -30mM imidazole, 50ml W2-40mM imidazole, and 20ml 80mM imidazole, and protein was eluted with 200mM imidazole (5ml) and 400mM imidazole (5ml). Then stripping was done to clean the beads for reuse and checked the purification quality by loading fraction collected at each purification step on 12% SDS PAGE gel.

3.2.14 Refolding, buffer exchange, and concentration of purified protein

Purified YchP OMP was refolded by rapid dilution. A total 10ml (3mg) of denatured purified YchP OMP was 12-fold diluted into 120 ml of dilution buffer containing 50mM Tris pH 7.5, 500mM NaCl, 5% glycerol, and 0.1% LDAO detergent and incubated for 2hrs with constant stirring using magnetic beads. Diluted protein was centrifuged at 11,000 rpm for 10min to check precipitation and then filtered using 0.22µm filters and then proceeded for buffer exchange and concentration using amicon of 10 MWCO. The refolded YchP OMP sample was loaded into the amicon and centrifuged at 4000x g until the volume of the protein in the amicon reaches 10 times lesser than the loaded protein. The volume was again made up to the initial volume using 50 mM Tris of pH 7.5, 500mM NaCl. Amicons were again centrifuged at 4000x g until the volume was reduced to 10 times lesser than the initial volume. The temperature was maintained 4° C throughout the procedure of buffer exchange and concentration.

Concentrated protein was then collected in the fresh autoclaved microcentrifuge tube and we proceeded with biophysical characterization.

3.2.15 Size exclusion chromatography for studying oligomerization

Size exclusion chromatography (SEC) can be used for studying dimer, trimer, or oligomer formation, it helps us in determining the absolute molecular weight and dimer, trimer, or oligomer state of protein. A Superdex 200 increase 10/300 GL column has been selected and provided a 0.4ml/min flow rate. Then washed the column with a 2-column volume of autoclaved, bath-sonicated distilled water, and then equilibrated the column with 1-column volume of equilibration buffer containing 50mM tris-Cl pH 8 and 500mM NaCl. Then 500µl refolded and concentrated ychP sample was injected into the column. For ychP only one sharp peak has been generated at 8ml retention volume, the peak was collected in fresh autoclaved microcentrifuge tubes and run the 12% semi-native PAGE gel to check whether the collected peak fraction for protein is a monomer, dimer, trimer, or oligomer.

3.2.16 Heat modifiability assay

Heat modifiability assay was used to check the folding and unfolding of the beta-barrel of the outer membrane protein. There will be a migration difference between folded and unfolded beta-barrel OMP. 2µg of folded beta barrel OMP was heat denatured by boiling at 95˚C for 5min. and both boiled and un-boiled samples were mixed with 2X SDS loading dye and loaded onto 12% semi-native PAGE gel and resolved at 150 voltages. The gel tank was kept in the ice bouquet.

3.2.17 Refolding study via tryptophan fluorimetry

Samples preparation- Sample tubes contained 20mM tris-Cl pH 8, 100mM NaCl, 20µg protein, and urea from 0M to 8M, and the rest volume was covered with autoclaved distilled water.

Blank preparation – 7 tubes with 1ml of the total volume of the blank have been prepared. Each tube contained 20mM tris-Cl pH 8, 100mM NaCl, and urea from 0 M to 8M, and the rest volume was covered with autoclaved distilled water.

The intrinsic tryptophan fluorescence has been recorded using a FluoroMax-4p spectrophotometer from Horiba Jobin Yvon (model: FM100). The excitation wavelength was set at 280nm with nm slit and emission spectra were measured between 310 to 450nm. Emission spectra were obtained when samples and blank were placed into a quartz tube with 1cm path length and 2nm bandwidth and then measured the fluorescence intensity was to investigate the impact of different urea concentrations (0-8M) on protein. Readings of the blank were subtracted from respected sample readings and then the emission spectra have been plotted. We have also studied the impact of detergents (LDAO and tween20) in 4x and 10x concentrations of CMC and lipids (DOPC and DMPC) with 0.25mM, 0.5mM and 1mM concentrations on 8M urea denatured YchP OMP and analyse the conformation changes by a tryptophan fluorimetry.

3.2.18 Secondary structure analysis via CD Spectroscopy

Samples preparation- Sample tubes contained 20mM tris-Cl pH 8, 100mM NaCl, 20µg protein, and urea from 0 M to 8M, and the rest volume was covered with autoclaved distilled water.

Blank preparation -7 tubes of blank for each sample has been prepared which contained 20mM tris-Cl pH 8, 100mM NaCl, and urea from 0 M to 8M, and the rest volume was covered with autoclaved distilled water.

Changes in secondary structure with increasing urea concentration (0M to 8M) have been analyzed using circular dichroism spectroscopy in a Jasco J-815 spectropolarimeter. CD spectra have been measured for each sample in the 190nm to 300 nm range. Blank and samples were placed into a CD cuvette with 1mm path length and 1nm bandwidth and measured the CD spectra one by one sequentially. We have also studied the impact of detergents (LDAO and tween20) in 4x and 10x concentrations of CMC and lipids (DOPC and DMPC) with 0.25mM, 0.5mM and 1mM concentrations on 8M urea denatured YchP OMP and analyze the conformation changes by circular dichroism and analyze the data using BeStSel tool.

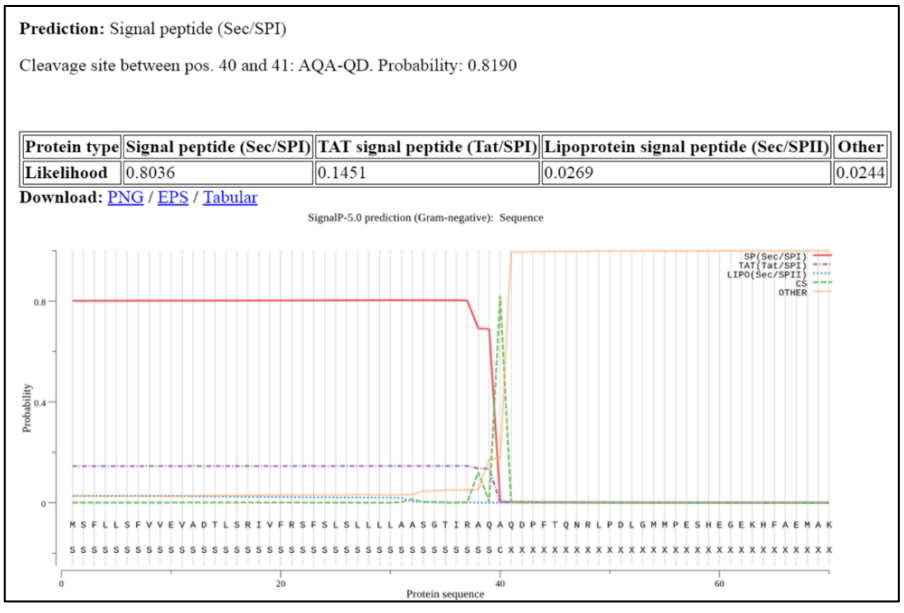
3.2.19 ATR-FTIR analysis

The secondary structure content of YchP OMP was further analyzed via ATR-FTIR spectroscopy. For protein, FTIR data shows a peak near 1700-1600cm^{-1} for the amide I region, and for the amide II region, it shows a peak around 1500-1400cm-1. So, the ATR-FTIR spectra were measured from 1300 to 1800 cm^{-1} for 100μg of YchP in a Tensor 27 (Bruker) FTIR spectrophotometer with a resolution of 2 cm^{-1}. Then the second derivative of absorption in the amide I region was taken to analyze the peak for secondary structure content in this amide I region and multiple fittings of each peak were deconvoluted using origin pro software and noted down the peaks and their respective wavenumber to analyze the secondary structure content in YchP.

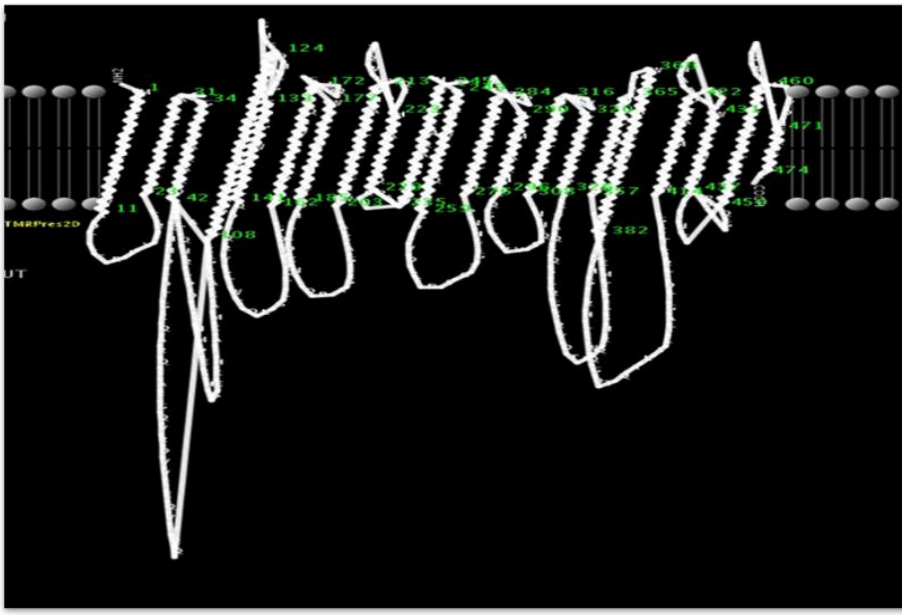
Chapter 4

In-silico study of YchP outer membrane protein from *Salmonella enterica serovar* Typhimurium

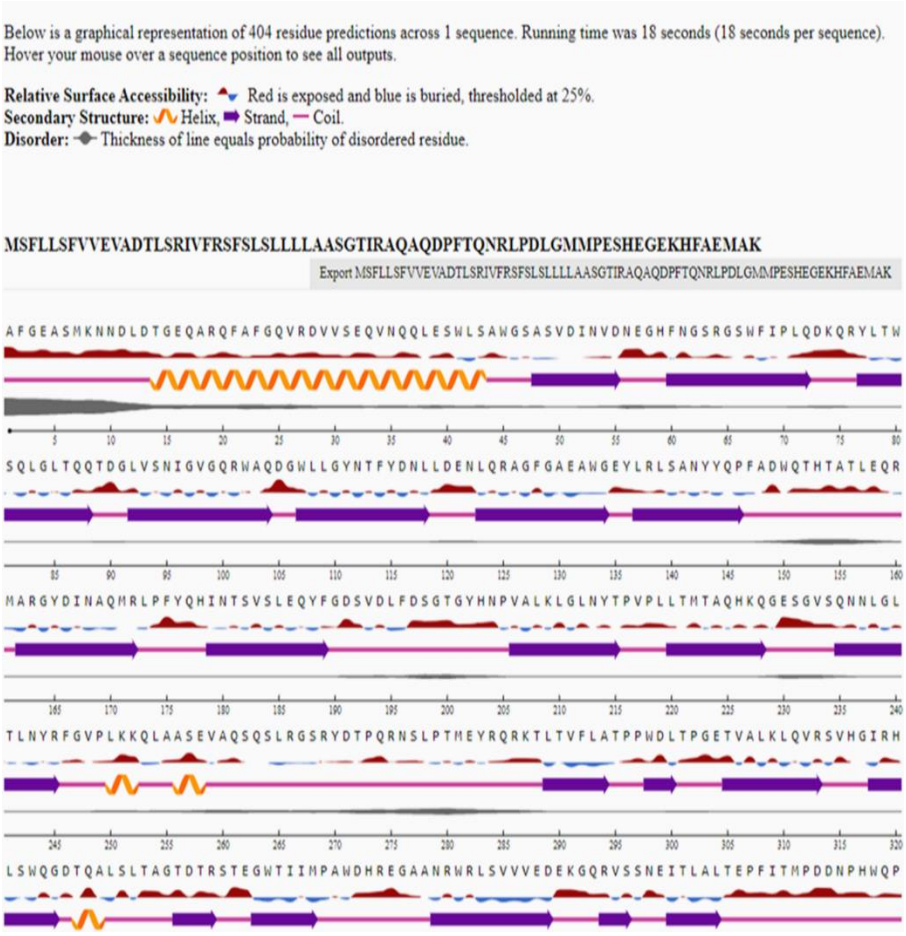
For YchP first, we used the OMP prediction tool such as PredTMBB and HhOMP which predict that there is a 97% probability of being an OMP for YchP and in YchP β strands spanning the transmembrane region as shown (Fig.5.1. B). As YchP is probably an OMP so it is expected to contain a signal peptide that was predicted using the SignalP5.0 tool which predicts the presence of signal peptides of 40 and 41 amino acid length (Fig.5.1. A). We have also used the NetsurfP2.0 tool for secondary structure prediction, the prediction result shows that YchP is composed of β strands, coils, and α helixes (Fig.5.1.C). We have also done tertiary structure prediction using the alpha-fold and I-taser tool, the prediction result shows that YchP is composed of around 14 β strands that form a β-barrel in topology (Fig.5.1. D and Fig.5.1. E) and contains 14 tryptophan residues.



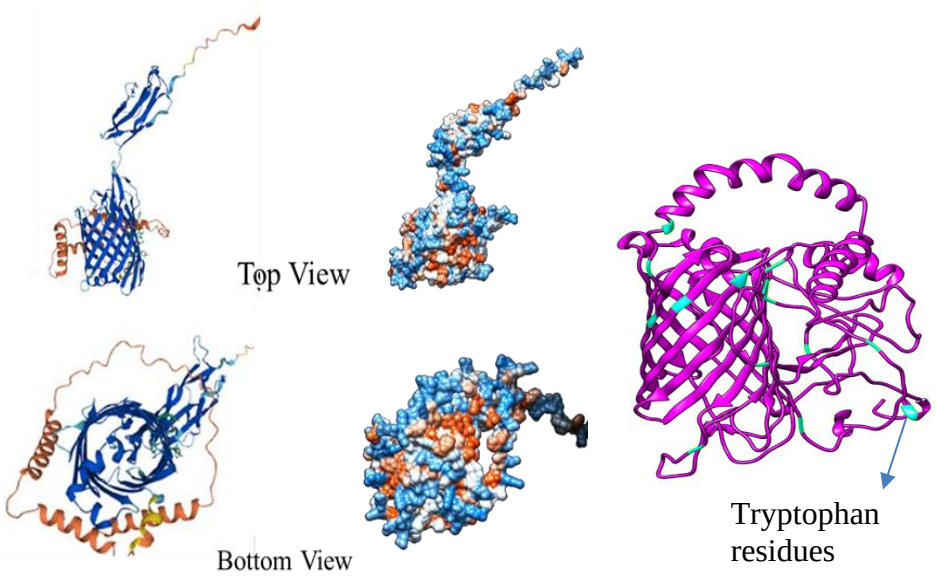
(a)



(b)


Below is a graphical representation of 404 residue predictions across 1 sequence. Running time was 18 seconds (18 seconds per sequence).
Hover your mouse over a sequence position to see all outputs.
Relative Surface Accessibility: Red is exposed and blue is buried, thresholded at 25%.
Secondary Structure: Helix, Strand, Coil.
Disorder: Thickness of line equals probability of disordered residue.
MSFLLSFVVEVADTLSRIVFRSFSLSLLLLAASGTIRAQAQDPFTQNRLPDLGMMPESHEGEKHFAEMAK
Export MSFLLSFVVEVADTLSRIVFRSFSLSLLLLAASGTIRAQAQDPFTQNRLPDLGMMPESHEGEKHFAEMAK

(c)



(d) **(e)**

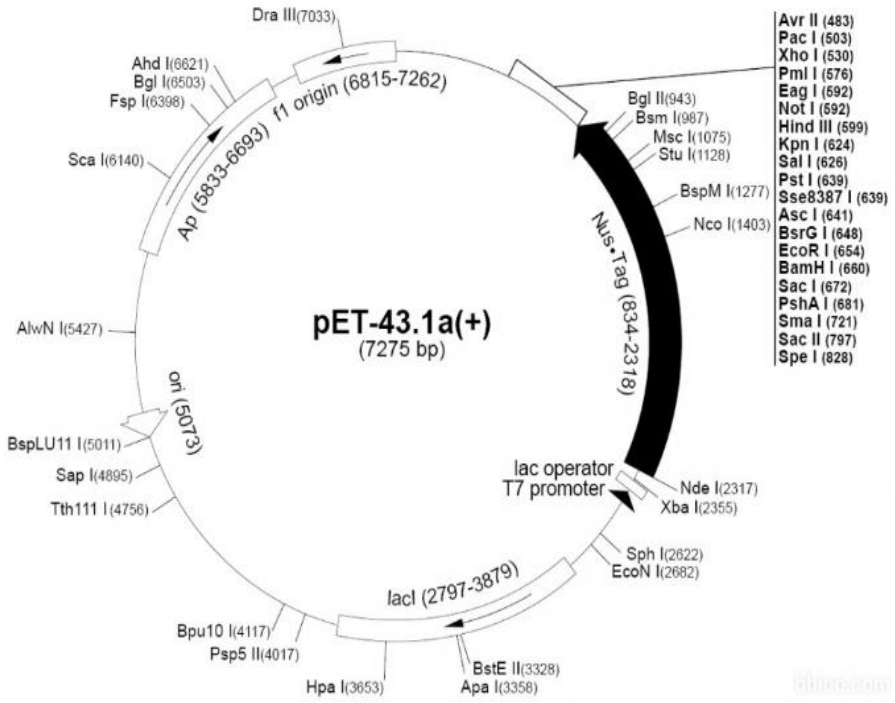
Fig.4.1 In-silico studies for YchP (a) Signal sequence prediction using SignalP-5.0 (b) PredTMBB For β barrel OMPs prediction (c) secondary structure prediction using NetsurfP2.0. (d) Alphafold-2.0 structure of YchP- Top View and Bottom View, (e) I-taster structure of YchP, the cyan blue color is indicating the total of 14 tryptophan residues in YchP OMP

Chapter 5

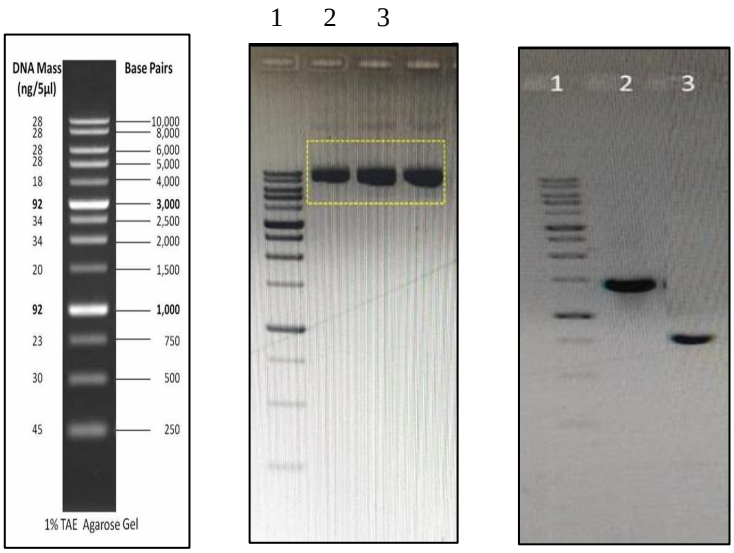
Results and discussions

5.1 *ychP* (from *Salmonella enterica* Typhimurium 14028s strain) cloning into pET-43a vector at *NdeI* and *Xho*I site

We have PCR amplified the *ychP* gene (1438 bp) from genomic DNA isolated from *Salmonella enterica* Typhimurium 14028s strain and performed restriction analysis for *ychP* PCR product, verification has done at *Hinc*II and *Ssp*I site. Then we cloned it into the pET-43a vector at *Nde*I and *Xho*I sites. For resultant clones, we have done colony PCR, recombinant plasmid PCR, and restriction analysis and confirmed them through DNA sequencing.

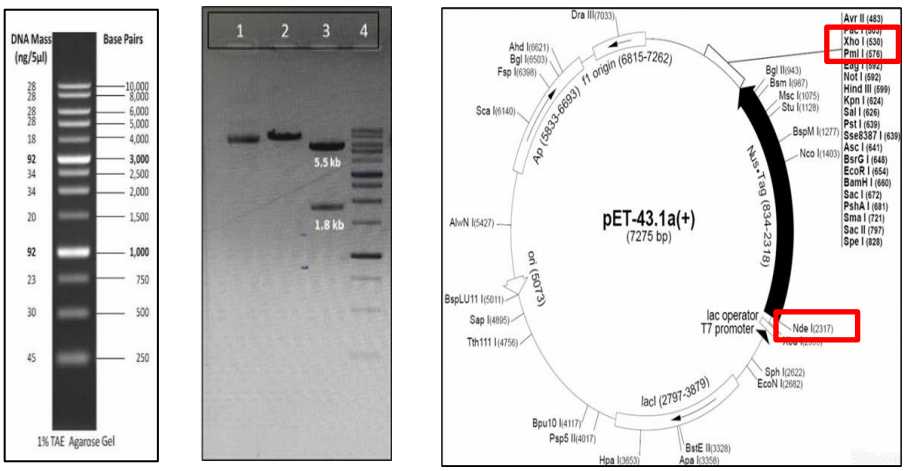


(a)



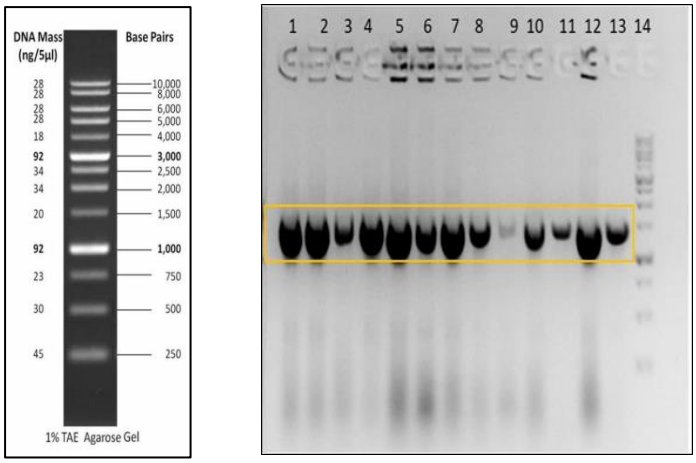
(b) **(c)** **(d)**

Fig. 5.1 (a) pET43a vector map (b) 1kb DNA ladder (1% TAE agarose) (c) gel image for isolated pET43a supercoiled plasmid, Lane 1- 1kb DNA ladder, Lane 2,3,4- Supercoiled pET43a vector (d) Gel image for insert preparation, Lane 1-1kb DNA ladder, Lane 2-PCR amplified *ychP* (1438 bp) with primers PK875F and PK876R and *Pfu* DNA polymerase.



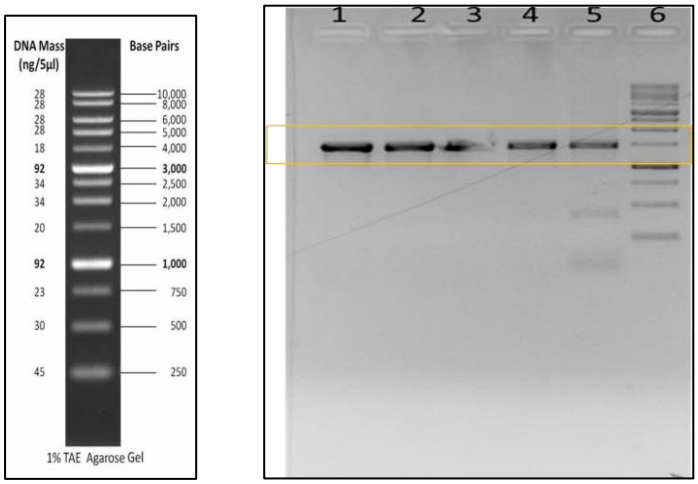
(a) **(b)** **(c)**

Fig.5.2 (a) 1kb DNA ladder (b) Gel image for restriction double digested pET43a vector, Lane 1- Uncut pET43a, Lane 2- Single digest of pET43a at NdeI site, Lane 3- Restriction double digested pET43a vector, Lane 4 -1kb DNA ladder



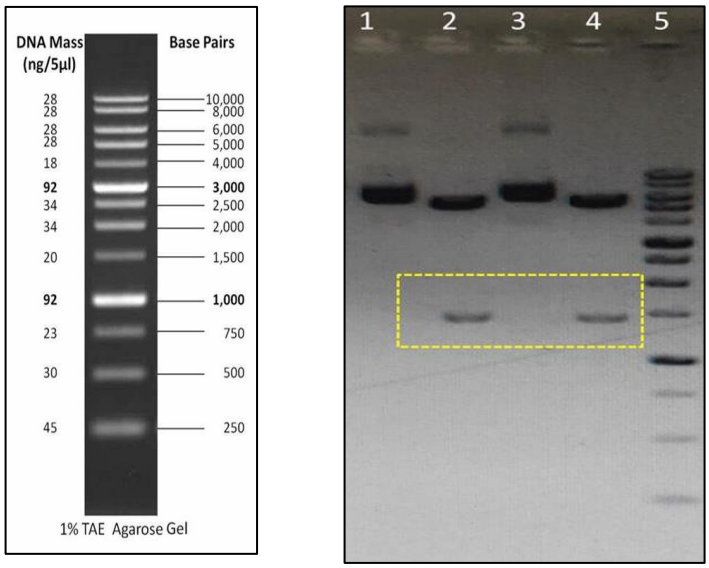
(a) **(b)**

Fig. 5.3 Gel images (a) 1kb DNA ladder (b) Colony PCR for r-*ychP* clones (*ychP* size- 1.4kb), Lane 1-12 -Clones For r-*ychP*, Lane 13- Positive control, Lane 14- 1kb DNA ladder



(a) **(b)**

Fig. 5.4 Gel images- (a) 1kb DNA ladder (b) Recombinant Plasmid PCR for r-*ychP*, *ychP* size-1438bp, Lane 1- r-*ychP* plasmid PCR products from clone no. 2,4, 5,6, 12, Lane 6. 1kb DNA ladder



(a) **(b)**

Fig. 5.5 Gel Images (a) 1kb DNA ladder (b) Confirmation of clones via restriction analysis for r-*ychP* plasmid, ychP – 1438bp, Double digested pET43a- 5.5 kb, Lane 1- r-*ychp* plasmid from clone 2, Lane 2- *Nde*I and *Xho*I digested r-*ychP* plasmid from clone 2, Lane 3- r-*ychp* plasmid from clone 4, Lane 4- *Nde*I and *Xho*I digested r-*ychP* plasmid from clone 4, Lane 5- 1kb DNA ladder.

5.2 Expression of pET43a-ychP-His

Resultant recombinant plasmids were transformed into Escherichia coli Rosetta (DE3) cells and induced with isopropyl-β-d-1-thiogalactopyranoside (IPTG). Induced cells were harvested, washed, and resuspended in 1ml of buffer containing 20 mM Tris-Cl, pH 7.6, NaCl 200mM, and 10% glycerol. Cell lysates were prepared and separated as supernatant and pellet, and supernatant and pellet samples were analyzed on 12% SDS-PAGE gels to check the expression of YchP outer membrane protein. YchP outer membrane protein size is 52kDa, the expression has been observed in the pellet or inclusion bodies with 1mM IPTG at 37°C and 220 rpm into *E. coli Rosetta* (DE3) cells and analyzed on 12% SDS-PAGE gel (Fig. 6.6).

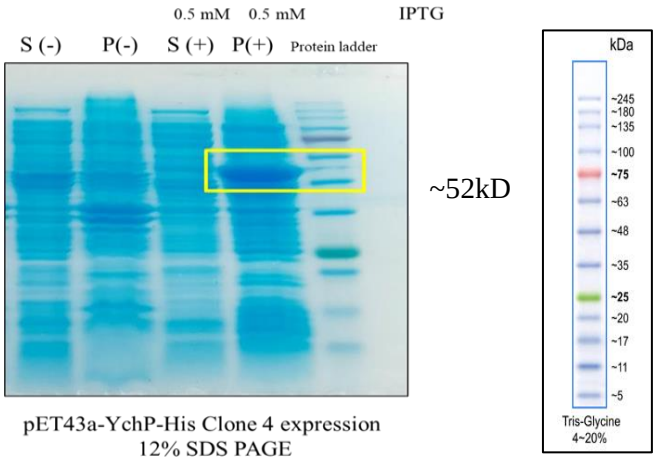


Fig.5.6 r-YchP expression-(a) Induction gel:0.5mM IPTG with 220rpm at 37°C for 4 hrs. Expressed protein present in the pellet (inclusion body) (b)tris-glycine protein ladder.

5.3 Solubilization and purification of r-YchP

For Solubilization induced pellet was resuspended with 20ml solubilization buffer of pH 7.5 containing 50mM tris-Cl pH 6.7, 500mM NaCl, and 8M urea, 20mM imidazole, and incubated at room temperature for 2-4 hrs. and then centrifuged at 12,000 rpm at 20°C for 30-40 min. Then purified using the Ni-NTA affinity column. YchP denatured OMP was eluted at 200mM and 400mM imidazole. (Fig. 6.7.)

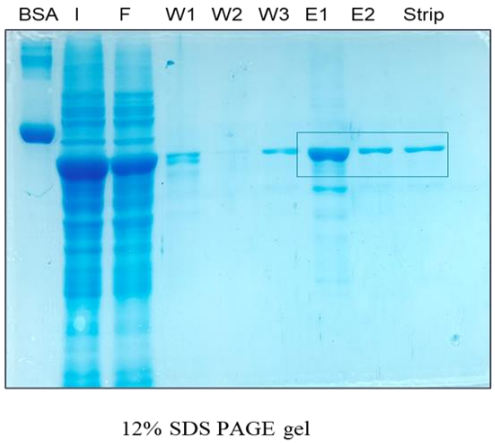


Fig. 5.7 Solubilization and purification of YchP: Lane 1 - BSA (5µg), Lane 2- Solubilized input (8M urea, 20mM tris (pH 7.4), 500mM NaCl, 20mM imidazole pH 8), Lane 3 – Flowthrough, Lane 4 - Wash 1 (30mM imidazole), Lane 5 - Wash 2 (40mM imidazole), Lane 6 - Wash 3 (80mM Imidazole), Lane 7 - Elution 1 (100mM imidazole), Lane 8 - Elution 2 (400mM imidazole), Lane 9 – stripping.

5.4 Refolding and concentration

Purified r-YchP OMP was refolded by rapid dilution. A total 10ml (3mg) of denatured purified r-YchP OMP was 12-fold diluted into 120 ml of dilution buffer containing 50mM Tris pH 8, 500mM NaCl, 5% glycerol, and 0.1% LDAO detergent and incubated for 2hrs with constant stirring using magnetic beads and then concentrated using Amicon of 10 MWCO (Fig. 6.8.).

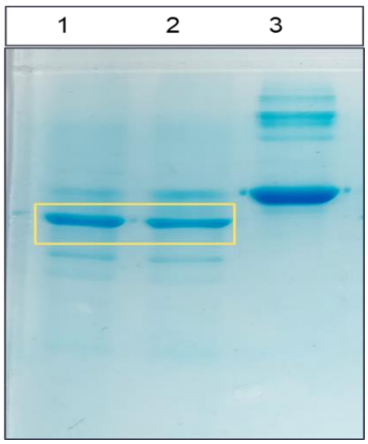
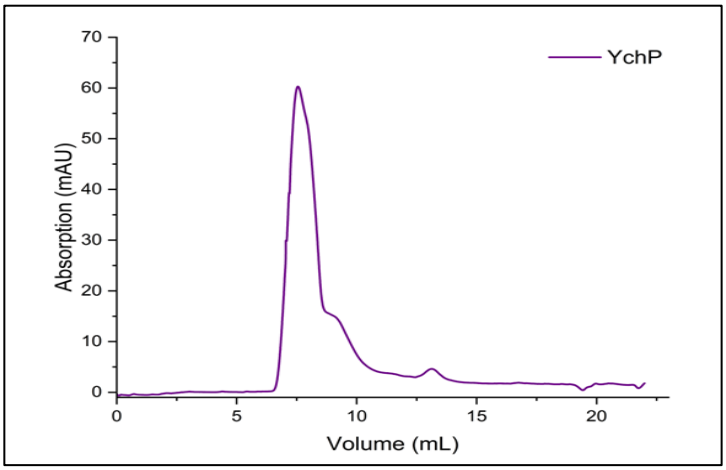


Fig. 5.8 Gel images for concentrated r-YchP OMP after refolding - Lane 1 and 2- refolded, concentrated r-YchP

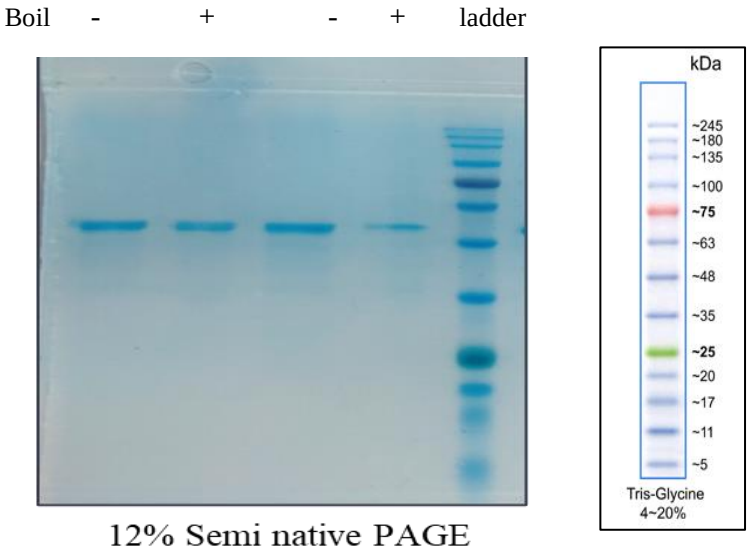
5.5 Biophysical characterization for expressed and purified r-YchP OMP

5.5.1 Size exclusion chromatography

Size exclusion chromatography (SEC) can be used for determining the absolute molecular weight and dimer, trimer, or oligomer state of the protein. A superdex 200 increase 10/300 GL column has been selected and provided a 0.4ml/min flow rate. Only one sharp peak for the YchP monomer of 60mAU has been generated at 8ml of retention volume (Fig. 6.9.a) and confirmation of monomer has been confirmed by running 12% semi-native PAGE gel (Fig.6.9. b).



(a)



(b) (c)

Fig.5.9 Size exclusion chromatography: (a) Size exclusion chromatogram, (b) 12% semi-native PAGE gel, (c) Protein ladder (gene direx).

5.5.2 Heat Modifiability Assay

YchP folded beta barrel OMP was boiled at 95˚C for 5min. and then boiled and un-boiled sample mixed with 2x SDS loading dye and loaded onto a 12% semi-native PAGE gel. There was no significant migration difference has been observed between boiled and un-boiled YchP beta barrel OMP. That shows the stable integrity of the beta-barrel of YchP OMP against heat.

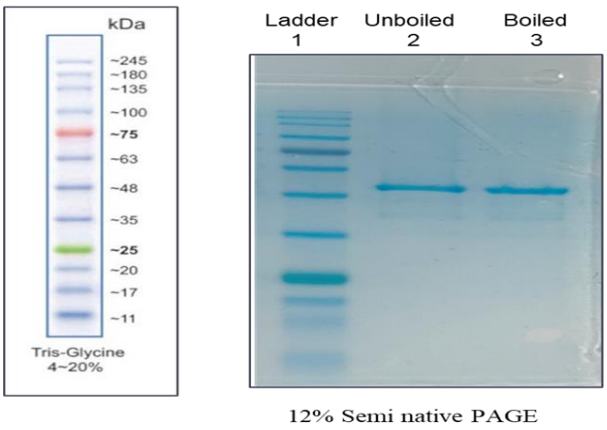
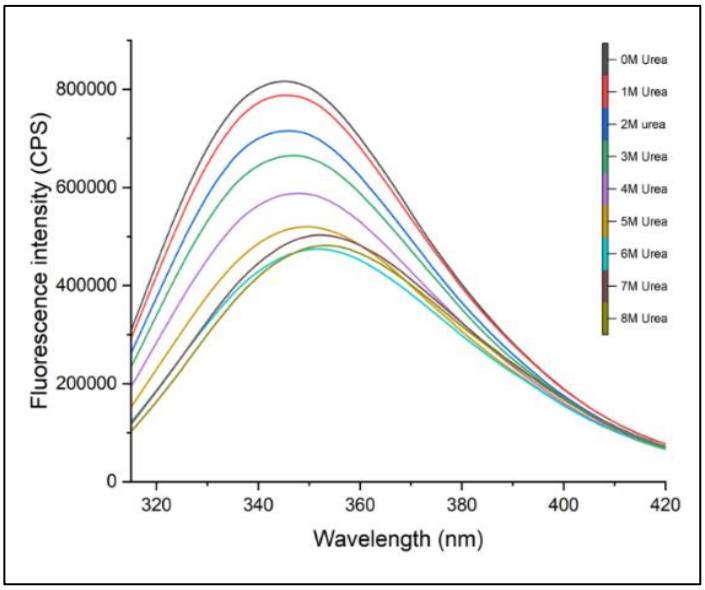


Fig.5.10 Heat modifiability assay for YchP beta barrel OMP

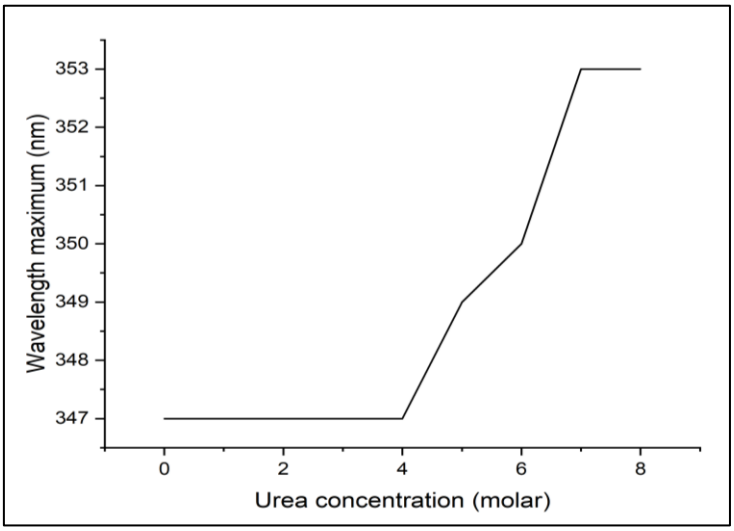
5.5.3 Tryptophan fluorimetry assay for YchP refolding study

Tryptophan is an aromatic amino acid which excited at 280nm wavelength and shows emission in the 305nm to 355nm range. The tryptophan emission extra has been measured to detect the changes in YchP OMP on denaturation with urea from 0M to 8M range. YchP contains 14 tryptophan residues. As the urea concentration increases the fluorescence intensity

decreases and after 4 M urea there is also a significant redshift of around 6nm in emission wavelength that has been observed that indicate the changes in YchP protein confirmation on denaturation with urea. The exposure of tryptophan residues varies due to this change in YchP confirmation on denaturation with increasing urea concentration.



(a)



(b)

Fig.5.11 YchP OMP refolding study by tryptophan fluorimetry assay (a) change in emission spectra of native YchP (0M urea) on increasing urea (denaturant) concentration (b) Denaturation kinetics curve

5.5.3.1 Impact of detergents and lipids on YchP topology via tryptophan fluorimetry assay

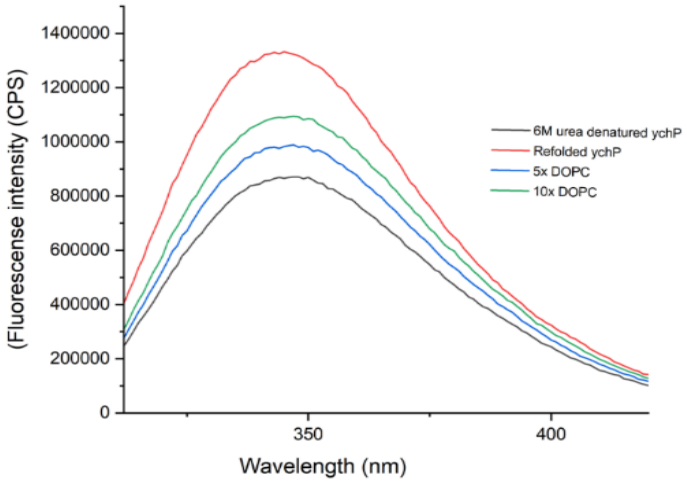
YchP contains 14 tryptophan residues. As we have studied that native YchP shows a 6nm red shift from 347nm, this shift is due to a change in YchP confirmation with the increase in urea concentration from 0m to 8M (fig.6.11). Similarly, for better understanding, we have also studied the refolding of denatured YchP in the presence of 4x and 10x of CMC of detergents (Tween 20 and LDAO) and lipids (DOPC and DMPC) via tryptophan fluorimetry assay. The sample was excited at 295nm wavelength and emission spectra were measured from 310-450nm wavelength. As shown in Fig. 12 denatured YchP shows a decrease in fluorescence intensity. But as the YchP was getting refolded it shows the blue shift in wavelength and increase in fluorescence intensity (fig.6.12) under different conditions of detergents and lipids. In the case of refolding study in the presence of 4x and 10x of CMC of DOPC, there is only a 1nm blue shift observed from 348nm (λ-max for denatured YchP) (fig.6.12a). While in the presence of 4x and 10x of CMC of DMPC, there is a 2nm blue shift observed from 348nm for denatured YchP, also a significant increase in fluorescence intensity has

occurred (Fig.6.12. b). In the presence of 4x and 10x of CMC of detergents Tween 20 and LDAO (fig. c and d), there is 4nm blue shift took place but Tween 20 shows fluorescence intensity much closer to denatured YchP (Fig.6.12.c) and LDAO shows a significant better increase in fluorescence intensity (fig, 6.12.d).

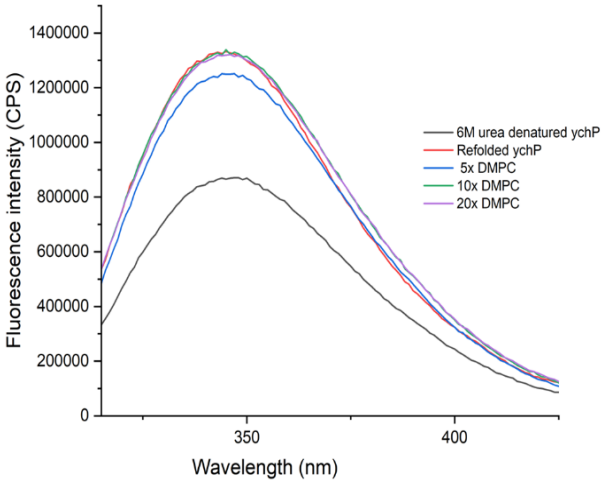
Based on the above tryptophan fluorimetry assay observation, the order of refolding capacity of used detergents and lipids for YchP are listed below;(table1.1)

Refolding capacity order	**Detergents and lipids**
High	DMPC, LDAO
Moderate	DOPC
low	Tween20

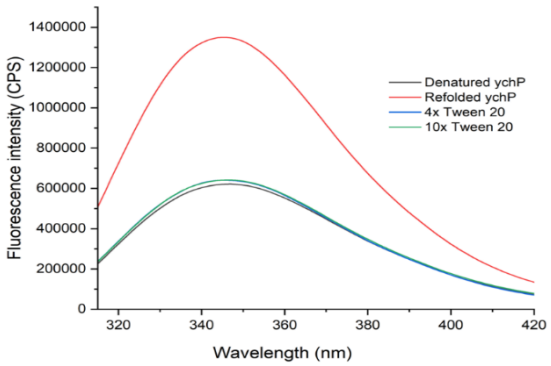
Table 1.1 order of refolding capacity of used detergents and lipids for YchP



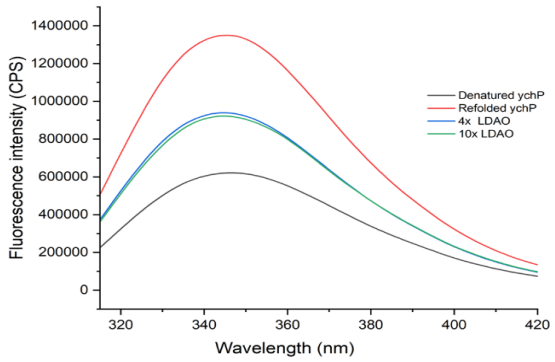
(a)



(b)



(c)



(d)

Fig. 5.12 Impact of detergents and lipids on YchP topology via tryptophan fluorimetry assay

(a) and (b) Tryptophan fluorescence spectra of denatured YchP:10 folds diluted in buffer containing DOPC and DMPC (0.25 mM and 1 mM) **(C) and (d)** Tryptophan fluorescence spectra of denatured YchP:10 folds diluted in buffer containing 4x and 10x of CMC of Tween 20 and LDAO.

5.4 YchP secondary structure analysis via circular dichroism spectroscopy

Changes in the secondary structure for YchP OMP with increasing urea concentration (0M to 8M) have been analyzed using circular dichroism spectroscopy in a Jasco J-815 spectropolarimeter. CD spectra have been measured for each sample in the 190nm to 300 nm range. In circular dichroism spectra, alpha-helical contents show positive peaks at around 192nm and negative peaks at around 222nm and 208nm, on the other hand, beta sheets show positive bands at around 198nm and negative bands at 216nm. Here in this experiment, blank samples were placed in a CD cuvette with 1mm (0.1cm) path length and 3nm bandwidth, and measured the CD spectra one by one sequentially. Obtained CD spectra show that for native YchP (0M Urea), there is one positive peak in the 192-222nm range and broad negative peaks between the 208-220nm range have been observed. That shows YchP protein consists of alpha helix and beta sheets in secondary structure content. Analysis of CD data reveals that native YchP contains 46.5% alpha helixes, 32.7% antiparallel beta sheets, and 20.7% parallel beta sheets in the secondary structure. As the urea concentration increases the denatured protein shows a shift in peaks due to disruption of native YchP secondary structure.

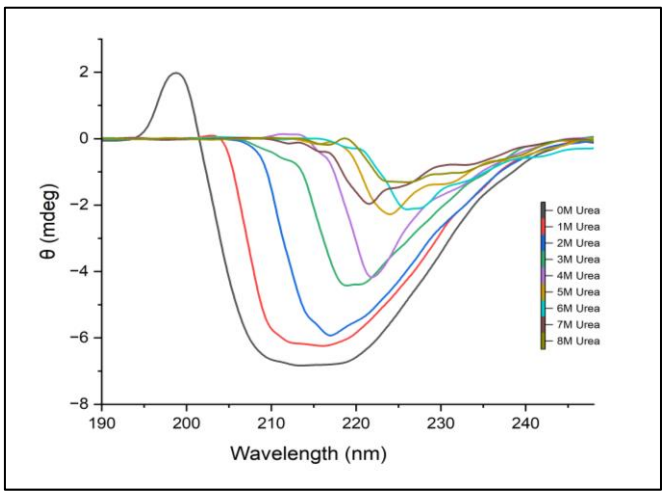
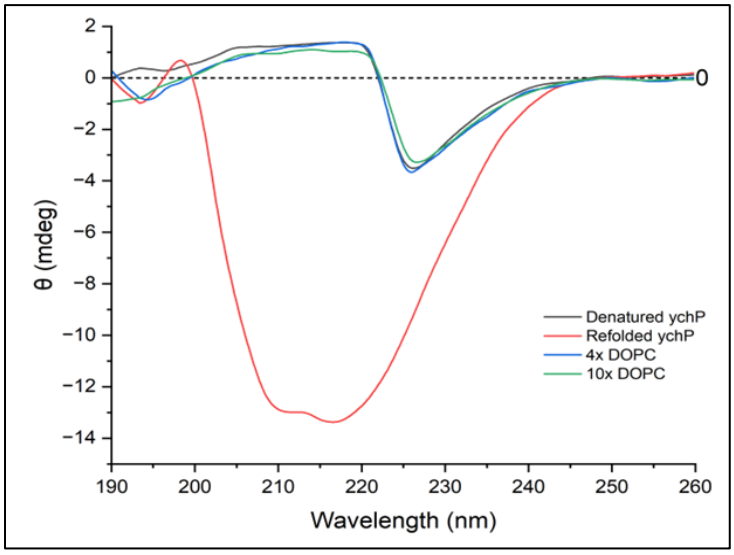


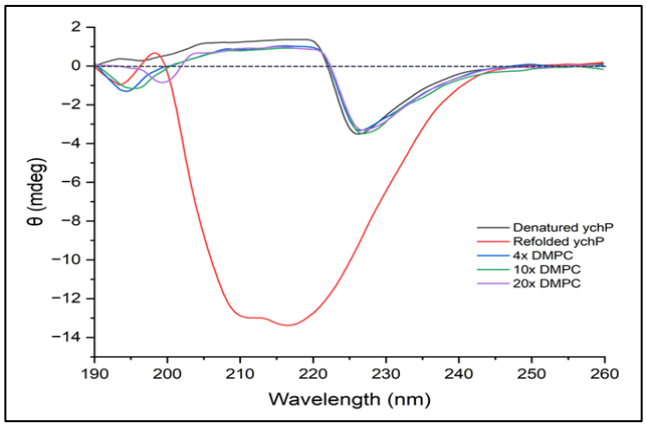
Fig.5.13 YchP OMP refolding study by CD spectra analysis

5.5.4.1 Impact of detergents and lipids on the topology of YchP via CD Spectroscopy

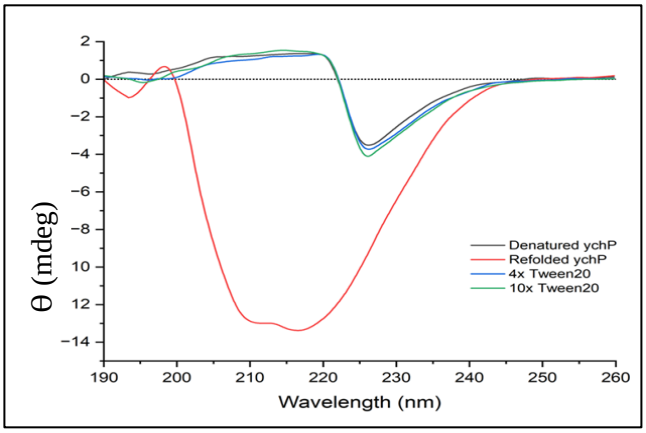
When detergents and lipids are used higher of their CMC values, it mimics the lipid bilayer membrane environment and will provide the conditions for refolding of membrane proteins. So here we have used 4x and 10x of CMC of lipids such as DOPC, DMPC, and detergents (LDAO and tween20), and measured the CD spectra for YchP under these different conditions from 190- 260nm wavelength and analyze the data using BeStSel tool, which shows the secondary structure elements presents in YchP under different buffer conditions (table 1.2, 1.3, 1.4, 1.5). CD spectra analysis of refolded YchP shows that it contains 46.5% alpha helixes, 32.7% antiparallel beta sheets, and 20.7% parallel beta sheets in secondary structure, and denatured YchP folded into random confirmation and beta sheets and alpha helix content decreases. When YchP I was refolded with 4x and 10x of CMC of lipids (DOPC and DMPC) and detergents (Tween 20 and LDAO), there are an increase in helix and beta sheets constituent has been observed and turns and other random structure decreases.



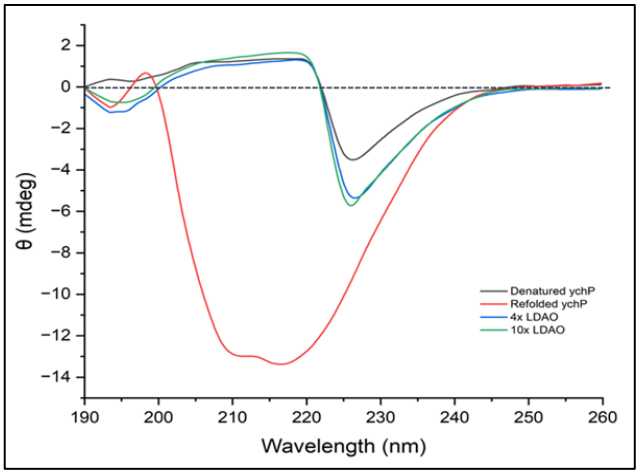
(a)



(b)



(c)



(d)

Fig. 5.14 Impact of detergents and lipids on YchP topology via CD spectroscopy (a) and (b) CD spectra for denatured YchP:10 folds diluted in buffer containing DOPC and DMPC (0.25 mM and 1 mM), (C) and (d) CD spectra of denatured YchP:10 folds diluted in buffer containing 4x and 10x of CMC of Tween 20 and LDAO.

Secondary structure	Denatured YchP (%)	Refolded YchP (%)	4x DOPC (%)	10x DOPC (%)
Helix	8.3	46.5	10.2	14.1
Antiparallel β-sheets	34.1	32.7	28.0	31.0
Parallel β-sheets	8.5	20.7	8.0	10.1
Turns	40	0	30.8	36.7
Others	9.1	0	23.0	8.1

Table 1.2 Analysis of percentage recovery of secondary structure content of denatured YchP with 4x and 10x of CMC of DOPC lipid via CD spectroscopy

Secondary structure	Denatured YchP (%)	Refolded YchP (%)	4x DMPC (%)	10x DMPC (%)	20x DMPC (%)
Helix	8.3	46.5	15.4	11.5	12.6
Antiparallel β-sheets	34.1	32.7	37.9	25.3	34.4
Parallel β-sheets	8.5	20.7	9.0	7.8	9.5
Turns	40	0	37.7	28.9	29.5
Others	9.1	0	0	26.4	14.0

Table 1.3 Analysis of percentage recovery of secondary structure content

Of denatured YchP with 4x and 10x of CMC of DMPC lipid by CD spectroscopy

Secondary structure	Denatured YchP (%)	Refolded YchP (%)	4x Tween20 (%)	10x Tween20 (%)
Helix	8.3	46.5	12.5	6.4
Antiparallel β-sheets	34.1	32.7	30.0	20.8
Parallel β-sheets	8.5	20.7	9.9	10.7
Turns	40	0	35.3	36.7
Others	9.1	0	12.3	25.4

Table 1.4 Analysis of percentage recovery of secondary structure content of denatured YchP with 4x and 10x of CMC of Tween20 detergent by CD spectroscopy

Secondary structure	Denatured YchP (%)	Refolded YchP (%)	4x LDAO (%)	10x LDAO (%)
Helix	8.3	46.5	4.8	0.9
Antiparallel β-sheets	34.1	32.7	35.9	36.4
Parallel β-sheets	8.5	20.7	19.1	21.2
Turns	40	0	40.2	41.5
Others	9.1	0	0.0	0.0

Table 1.5 Analysis of percentage recovery of secondary structure content of denatured YchP with 4x and 10x of CMC LDAO detergent via CD spectroscopy.

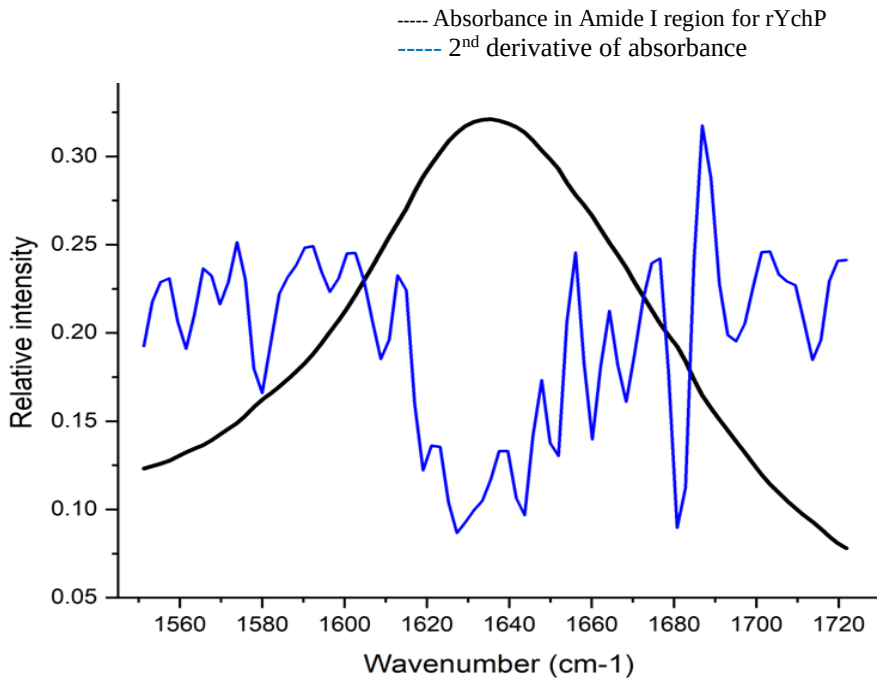
Refolding capacity order of used detergents and lipids based on CD spectra analysis for YchP refolding study

Refolding capacity order	Detergents and lipids
High	LDAO
Moderate	DMPC, Tween 20
low	DOPC

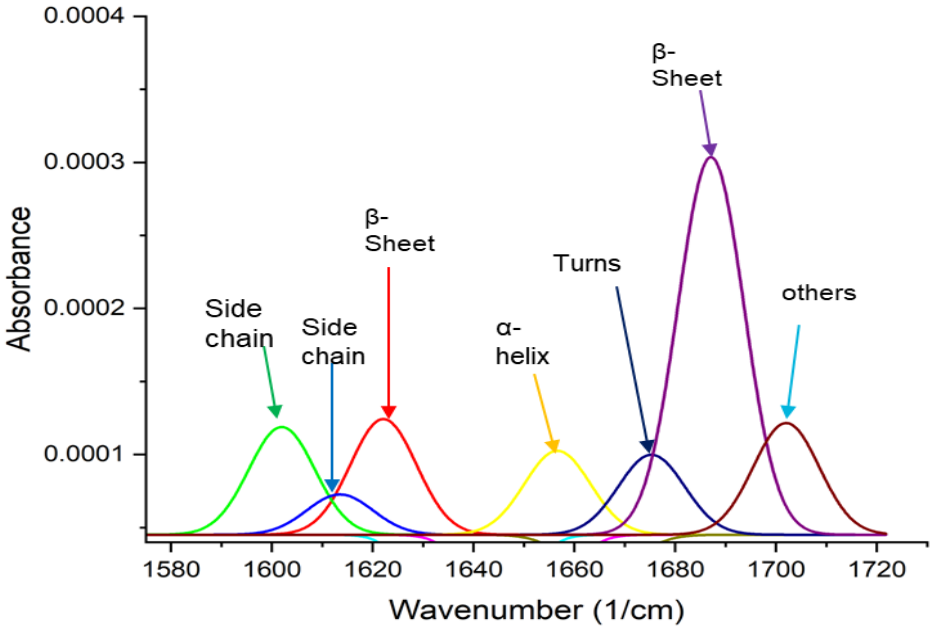
Table 1.6. Refolding capacity order of used detergents and lipids based on CD spectra analysis

5.5.5 Secondary structure study of YchP via ATR-FTIR Spectroscopy

ATR-FTIR spectra for YchP from 1600-1700cm^{-1} range contain the amide I region band. The observed wave frequencies, on deconvolution of multiple fitting, in this amide I region were identified for secondary structure composition in YchP **(Table 1.7).**



(a)



(b)

Fig. 5.15 ATR-FTIR analysis for YchP in the amide I region band from 1600-1700cm-1 (a) FTIR spectra after taking 2nd derivative absorbance in the amide I region band (b) FTIR spectra after deconvolution of absorption peaks observed in graph**.**

Secondary structure components	**Wavenumber (cm^{-1})**
Sidechain	1601.80 and 1612.55
Beta sheets	1622.11 and 1686.64
Alpha helix	1655.74
Turns	1675.54
others	1700

Table 1.7. Secondary structure composition in YchP based on peak observed at the corresponding wavenumber.

Discussions

OMPs in gram-negative bacteria play so many roles like transportation, host-pathogen interaction, causing virulence, etc. As the structure of the molecule determines its function, so understating of structural aspects of OMPs is crucial to understand its functional role. YchP OMP (around 52kDa) from *Salmonella enterica serovar* Typhimurium belongs to the invasin family OMP, which helps bacterial to enter host mammalian cells to infect the host since it is having a strong affinity to β-integrin present in the mammalian host cell surface. We performed the cloning, expression, purification, and solubilization of YchP OMP and refold it via the rapid dilution method.

We performed the in-silico study for YchP OMP to predict the presence of signal peptide, which was around 41 amino acid residues long (fig.5.1 a) We have also used various tools for secondary and tertiary structure prediction of YchP such as netsurfP2.0, alpha fold and I-tasser, which shows that YchP comprised of alpha helix, beta sheets, and coils **(**Fig.5.1 c, d & e**)** and alpha fold and I-taser predicted tertiary structure for YchP shows that YchP is a β-barrel OMP (Fig.5.1 d &e). YchP shows 37.55% similarity to transmembrane β-domain from invasin from *Yersinia pseudotuberculosis* (PDB DOI: https://doi.org/10.2210/pdb4E1T/pdb) which involves adhesion and invasion into the host cell. YchP contains 14 tryptophan residues, which is good enough for fluorometric assays. After in-silico analysis, we have done cloning, and expression in the *E. coli* host and then performed purification, solubilization, and refolding of YchP.

We have performed a biophysical characterization study for a few aspects of YchP. Size exclusion chromatography for YchP and semi-native PAGE gel analysis for peak collected in SEC chromatogram shows that YchP exists in monomeric form (fig.6.9). Heat modifiability is a unique feature for β-barrel OMP, that shows migration difference between heat denatured and native β-barrel OMP. YchP did not show any migration difference between native and heat-denatured YchP

(fig.6.10), which can be due to the heat stability of the YchP beta-barrel. We have done refolding study for YchP using tryptophan fluorimetry assay and CD spectroscopy in the presence of denaturant (urea). Refolding study for YchP via tryptophan fluorimetry in the presence of 0 to 8M urea shows that 6nm λ_{max} shift (red shift) is there beyond 4M urea, which is due to the change in YchP confirmation **(**fig.6.11), in presence of urea denaturant that cause different exposure of tryptophan residues to the environment. CD spectra analysis for native YchP shows that YchP consists of 46.5% alpha helixes, 32.7% antiparallel beta sheets, and 20.7% parallel beta sheets in secondary structure and it gives a positive peak near 198nm and a broad negative peak between 210-220nm (fig.6.13). As the concentration of urea denaturant increases from 0 to 8 M, there is a shift in peak observed due to the denaturation of YchP native confirmation (fig.6.13). We have also studied the impact of detergents and lipids on YchP renaturation. Here we have used 4x and 10x of CMC of detergents LDAO and Tween20 and lipids DOPC and DMPC. Tryptophan fluorimetry study and CD spectra analysis show that LDAO and DMPC form the favourable environmental conditions for YchP refolding, where DOPC and Tween20 are the least favourable. LDAO, DMPC, and DOPC favour increasing the antiparallel β-sheet content in YchP (Fig.6.14). ATR-FTIR analysis for YchP shows that YchP is composed of β-sheets (predominantly), α-helixes, and turns (Fig.6.15).

YchP is a putative invasin OMP, which has an affinity to β-integrin to invade the host mammalian cell to cause infection. Its further biophysical analysis can help us to better understand its role and molecular mechanism behind the pathogenesis caused by *Salmonella*.

Chapter 6

Conclusion and scope for future work

Salmonella enterica serovar Typhimurium is the major global key reason for diarrhoeal diseases. Here we have done cloning expression of YchP (putative invasin) OMP) from *Salmonella* into *E. coli* host and biophysical characterization of YchP OMP. In- silico study for YchP shows that it is a β- barrel OMP, containing 41 amino acids residues long signal peptide. YchP is majorly composed of β-sheets in a secondary structure and localized in the outer membrane region. SEC and semi-native PAGE gel analysis for YchP shows that YchP exists in monomeric form, which is resistant to heat denaturation since it did not show heat modifiability. Refolding study of YchP via tryptophan fluorimetry and CD spectra analysis shows that from 4M urea, YchP starts to unfold. A study of renaturation of YchP in the presence of 4x and 10x of CMC of detergents (LDAO and tween20) and lipids (DOPC and DMPC) shows that LDAO and DMPC provide favourable conditions to refold YchP compare to DMPC and Tween20. CD spectra analysis and ATR-FTIR spectra analysis for YchP shows that YchP is comprised of α-helix, β-sheets, and turns. CD spectra analysis shows that YchP contains 46.5% alpha helixes, 32.7% antiparallel beta sheets, and 20.7% parallel beta sheets in the secondary structure.

These structural features of YchP OMP may give us better insight into understanding the molecular mechanism behind virulence caused by *Salmonella* and may help us to develop a treatment against it to treat the infection caused by *Salmonella.*

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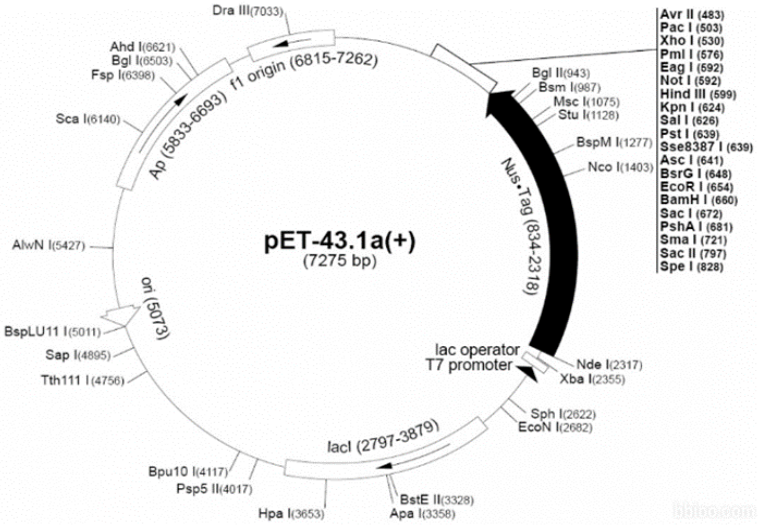
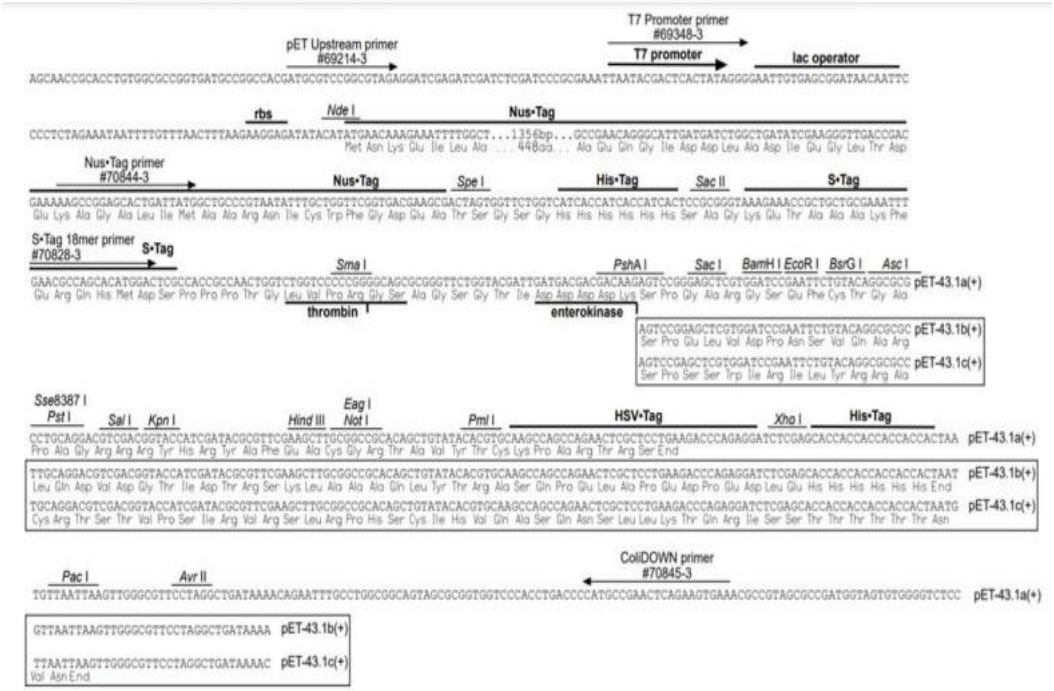
Annexure

7.1 List of primers for cloning of YchP into pET43a

Primer	Sequence (5'-3')	Restriction site
PK923F	CTGG**CATatg**tcattcctcttatcttttgttgtagaag	*Nde*I
PK924R	GAA **CTC GAG** ttgctcctggaacggttg	*Xho*I

7.2 Plasmid map of pET43a





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