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Cloning, Expression, Purification of the SARS- CoV- 2 Envelope Protein

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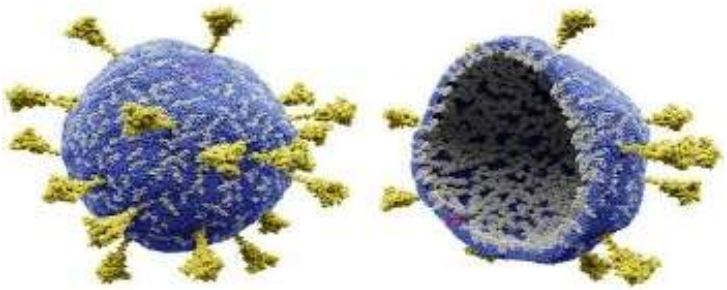


Abstract:
The severe acute respiratory pattern coronavirus 2(SARS-CoV- 2) surfaced as the causative agent of the COVID-19 epidemic, fleetly spreading among humans. This contagion enhances its infectivity and pathogenicity by interacting with host proteins, eased by its viral proteins binding to mortal cellular targets. Among these, the Envelope(E) protein plays a vital part, particularly through its C-terminal DLLV motif, which binds to a hydrophobic fund formed by the PDZ and SH3 disciplines of PALS1. This commerce contributes to the E protein's part in viral pathogenicity. This study explores the eventuality of dismembering the commerce between the PALS1 protein and the E protein using peptides and small motes, aiming to reduce viral infectivity. To probe the part of the E protein, a PCDNA- ENV plasmid was used to reduplicate and express the protein in a bacterial BL21 system. These findings give perceptivity into remedial strategies targeting mechanisms intermediated by the SARS-CoV- 2 E protein.

Keywords;SARS- CoV- 2, Envelope protein, COVID- 19, ACE2 receptor, PALS1 commerce, DLLV motif, Ni- NTA affinity chromatography, remedial targets.

Introduction:

COVID-19 has surfaced as an agony worldwide. The etiological agent of this complaint is an enveloped, single-stranded positive-sense RNA contagion, called the SARS-CoV-2. Since the outbreak of this contagious complaint in 2019, from Wuhan, China, millions of deaths have been reported by the new coronavirus. The complaint causes mild to severe respiratory problems, pneumonia, fever, cough, headache, anosmia or hyposmia, gastrointestinal problems, or indeed death in severe cases. SARS-CoV-2- 2 is a member of the order Nidovirales, coronaviridae family, rubric Betacoronavirus, and the species Severe Acute Respiratory Syndrome Coronavirus.

It's set up that the SARS- CoV- 2 genome is phylogenetically related to SARSCoV(79.6) and MERS- CoV(50), having a length of roughly 30 kilobases. Two incompletely lapping open reading frames 1a and 1b present in the 5' region of the genome get restated into large polypeptides pp1a and pp1b upon infection. Viral enzymes, the proteases further stick the two polyproteins to produce 16 functional non-structural proteins(NSPs), from nsp1 to nsp16. The remaining one-third part of the viral genome possesses ORFs garbling structure-forming proteins like Nucleocapsid, membrane protein, envelope protein, or shaft proteins.

Materials And Methods:

PCR reaction mixture:

PCR amplification of SARS-CoV-2 from pcDNA3.1 vector construct

The pcDNA3.1 glycerol stock was taken, and plasmid was isolated. From this plasmid, SARS-CoV-2 Env protein was amplified from 0.3 µ1 (100ng/µl) of pcDNA3.1 vector construct with primers having restriction sites BamH1 and EcoR1 in forward and reverse primers respectively.

The mixture was kept in Gradient Polymerase Chain Reaction and was run according to the following program.

Steps	Temperature	Time	Cycles
Initial denaturation	95	5 min	1X
denaturation	95	30 sec	30X
Annealing	60	30 sec	
Elongation	72	30 sec	
Final Elongation	72	5 min	1X
Hold	4	∞	_

DEPC water Taq	6.38µl
buffer dNTPs	2µl
Forward primer	0.2µl
Reverse primer	0.2µl
Taq polymerase	0.2µl
Template	0.2µl
	0.22µl

The reaction product was then run on 1% agarose gel to detect the amplicon and the resultant amplicon was extracted using a Thermo Scientific Gel Extraction kit.

Double digestion of SARS-CoV-2 ENV amplicon (insert) and Pet28:

a (Vector):

The extracted SARS-CoV-2 Env amplicon and Pet 28 a vectorwere digested with restriction enzymes BamHI and EcoR1.

Restriction Digestion Mixture-

	Insert	**Vector**
DEPC water	7.47 µl	8.64 µl
Cut smart buffer	1.5 µl	2.5 µl
Bam H1 EcoR1	0.33 µl	0.33 µl
Plasmid	0.33 µl	0.33 µl

	5.37 µl	3.20 µl
Total digestion Mixture	15 µl	15 µl

The digestion mixture was kept at 37°C for 3 hours. It was then run on 0.8% agarose gel and the digested bands were gel extracted using a Thermo Scientific Gel Extraction Kit.

Ligation of digested SARS-CoV-2 Env amplicon (insert) and pET28 a Vector

The digested SARS-CoV-2 Env amplicon (insert) and Pet28 a vector was ligated using T4 ligase enzyme using a vector: insert ratio of 1:5.

Ligation Mixture-

DEPC water -13.8 µl
Ligase buffer -2 µl
Ligase enzyme -2 µl
Pet28a vector -1.97 µl
SARS-COV-2Env insert -0.23 µl
TOTAL MIXTURE LIGATION -20

Preparation of DH5α Competent Cells and Transformation of the Ligation Product(Env/ pET28a Construct)

1. **Overnight Culture;**A primary culture of DH5α bacteria was grown overnight in an LB medium.
2. **Fresh Folklore**; 200 mL of the late culture was transferred to 200 mL of fresh LB medium and incubated in a shaker at 37 °C until the optic viscosity(O.D.) at 600 nm reached 0.5.
3. **Cooling Step**; 50 mL of the culture was transferred to a Falcon tube and stupefied on ice for 20 twinkles.
4. **Centrifugation**; The cells were agglomerated by centrifuging at 8,000 rpm for 15 twinkles at 4 °C.
5. **$MgCl_2$ Treatment**; The bullet was resuspended in ice-cold 0.1 M $MgCl_2$.
6. **Alternate Centrifugation;** The suspense was centrifuged at 6,000 rpm for 15 twinkles at 4 °C**.**
7. **$CaCl_2$ Treatment;** The supernatant was discarded, and the bullet was gently dissolved in 0.1 M $CaCl_2$.
8. **Cold Incubation**; The suspense was incubated at 4 °C for 1 – 2 hours to enhance faculty, after which the cells were agglomerated again by centrifugation.

9. **Final Resuspension**; The bullet was resuspended in 2.5 mL of ice-cold 0.1 M $CaCl_2$ containing 15 glycerol.
10. **Aliquoting and Storage**; Aliquots of 200 µL were prepared in sterile Eppendorf tubes and stored at 80 °C for unborn use.

Transformation of Ligation Product(pET28aEnv) into DH5α Cells :

Protocol:

One vial of competent cells was taken out from 80 °C and kept on ice for 20- 30 twinkles. 10 µl of ligation product was added to the cells, mixed gently, and left on ice for 20- 30 twinkles. The cells were subordinated to heat shock at 42 °C for 90 seconds.The Eppendorf tube was incontinently transferred on ice and kept for 1 nanosecond.900 µl of fresh LB media was added and mixed well.The tubes were incubated at 37 °C in an incubator-shaker for 1- 2 hours. The tube was subordinated to centrifugation at 8000 rpm for 1 nanosecond .The supernatant (900 µl) was discarded.

The bullet was re-suspended in the remaining supernatant and 80 µl was plated on a LA Kanamycin plate. The plate was kept in a 37 °C incubator overnight. Colony PCR of transformed cells

Colony PCR of transformed cells was performed with the same primers used for the PCR amplification. 6 colonies along with control were subjected to PCR.

Colony PCR Reaction Mixture-

Master mix	5 µl
Forward primer	0.2 µl
Reverse Primer	0.2 µl
DEPC water	4.6 µl All
Colony	
Total reaction mixture	10 µ1

The mixture was kept in a thermal cycler Polymerase Chain Reaction was run according to the following program.

PCR program –

Steps	Temperature	Time	Cycles
Initial Denaturation	95	5 min	1X
Denaturation	95	30 sec	30X
Annealing	60	30 sec	
Elongation	72	30 sec	
Final Elongation	72	5 min	1X
Hold	4	∞	_

The reaction product was then run on 1% agarose gel.

Cover of plasmid from changed-over cells utilizing Thermo Logical GeneJET Plasmid Miniprep Unit

1. 10 ml of LB was contributed with a single colony and brooded at 37 °C overnight.
2. The pelleted cells were resuspended in 250 µL of the Resuspension Arrangement.
3. The cell tension was exchanged to a microcentrifuge tube.
4. The microscopic organisms ought to be suspended completely by pipetting up and down until no cell clumps stay.
5. 250 µl of the lysis result was included and blended totally by flipping the tube 4- 6 times until the result came thick and somewhat clear.
6. 350 µL of the Neutralization result was included and blended incontinently and totally by flipping the tube 4- 6 times.
7. Centrifuge for 5 min to bullet cell flotsam and jetsam and chromosomal DNA.
8. The supernatant was exchanged to the GeneJET turn column by emptying or pipetting.
9. Exasperating or exchanging the white accelerate was maintained at a strategic distance from.

10. Centrifuged for 1 min. The influx-through was disposed of and the column was set back into the same collection tube.
11. 500 µL of the Wash Arrangement was included to the Quality spurt turn column.
12. Centrifuged for 30- 60 seconds and the influx-through was disposed of. The column was set back into the same collection tube.
13. Washing was rehashed utilizing 500 µL of the Wash Arrangement.
14. The influx-through was disposed of and centrifuged for a new 1 min to expel the remaining Wash Arrangement. This step is basic to dodge leftover ethanol in plasmid preparations.

The quality spurt turn column was exchanged into a new 1.5 ml microcentrifuge tube. 20µL of the Elution Buffer was included in the center of the GeneJET turn column film to elute the plasmid DNA.
Incubated for 10 min at room temperature and centrifuged for 1 min.

- The elution step was repeated in other microcentrifuge tubes with the same spin column and 20µl of elution buffer.
- The column was discarded, and the purified plasmid DNA was stored at -20°C after measuring the concentration.

Gel Electrophoresis:

In order to confirm the presence of plasmid, agarose gel electrophoresis was done from the isolated plasmid.

- **Preparation of BL21Competent cells:**
- The primary culture of bacteria BL21 was grown overnight in LB media.
- 200ml of primary culture was taken and added to the fresh 200ml of LB media and kept in a shaker incubator at 37˚ C until the O.D. reached 0.5.
- 50 ml was transferred to a falcon tube and incubation was done in ice for 20 min.
- Centrifugation was done at 8,000 rpm at 4°C for 15 min.
- Resuspended on ice with 0.1 M MgCl2.
- Centrifugation was done at 6,000 rpm at 4°C for 15 min.
- The supernatant was discarded 0.1M CaCl2 was added and the pellet was dissolved.
- The dissolved pellet was left for 1-2 hours at 4°C and the cells were pelleted down.
- The supernatant was discarded and the pellet was resuspended in 2.5ml of 0.1M CaCl 2 and 15% glycerol.
- 200 µl aliquots were made in autoclaved Eppendorf tube and were stored at 80°C.

Transformation of pET28a Env into BL21 competent cells:

1. Plasmid was transformed into BL21 competent cells.
2. Competent cells were kept on ice for 20- 30 twinkles.
3. 1µl of asked DNA was mixed and left on ice for 30 twinkles.
4. Heat shock was given for 2 twinkles at 42 ˚C.
5. The sample was kept on ice again for 1 nanosecond.
6. 800µl of fresh LB media was added.
7. The sample was mixed and incubated at 37 ˚ C under shaking for 1 hour.
8. Centrifugation was done at 6000 rpm for 1 nanosecond.
9. 900µl of the supernatant was discarded.
10. The bullet was suspended and plated on LB agar containing kanamycin and incubated overnight.
11. The colonies attained were again renovated onto a kanamycin plate.

Overexpression of recombinant SARS- CoV Env protein

To induce the expression Env with the 6XHis label using the expression plasmid vector, Pet28a Env, the following protocol has been followed

a) 5 ml of primary culture was invested in LB medium with specific antibiotics kanamycin overnight at 37 °C.

b) The coming day 50ul of primary culture was invested into 5 ml of LB medium and incubated at 37 °C, 190 rpm till O.D 600nm reached 0.5- 0.6.

c) 5 µl of 1M IPTG was added to the culture to get the final attention of 1mM.

d) The cells were allowed to grow at 18 °C for 16 hrs following which they were collected.

e) 500ul of cells were taken in a 1.5 ml microcentrifuge tube and 1x lading color was added.

This mixture was boiled and then loaded onto the 12% SDS gel to visualize the overexpression of Env protein against uninduced as a negative control.

Purification of recombinant protein using Ni-NTA column chromatography:

A. **Proteinexpression**:

To induce the expression Env with the 6XHis tag using the expression plasmid vector, Pet28a, the following protocol has been followed:

5 ml of primary culture was inoculated in LB medium with specific antibiotics Ampicillin overnight at 37°C. The next day 2ml of primary culture was inoculated into 200ml of LB medium and incubated at 37°C, 190 RPM till O.D 600nm reached 0.5- 0.6.100µl of 1M IPTG was added to the culture to get the final concentration of 1mM.The cells were allowed to grow at 18°C for 16 hrs following which they were collected. Cells were harvested by centrifugation at 10,000 rpm for 10 minutes at 4°C.The supernatant was discarded, and the pellets were used for protein purification.

B. Cell Lysis:

5 ml of lysis buffer was added to the pellets for their dissolution. Sonicated the solution in ice for 15 minutes using 10 seconds of bursts at 35% amplitude with 10 second cooling period between bursts. The process was repeated until a clear lysate was visible. The lysed cells were centrifuged at 10500 rpm for 50 minutes at 4°C. The supernatant was collected which consists of the cell lysate.

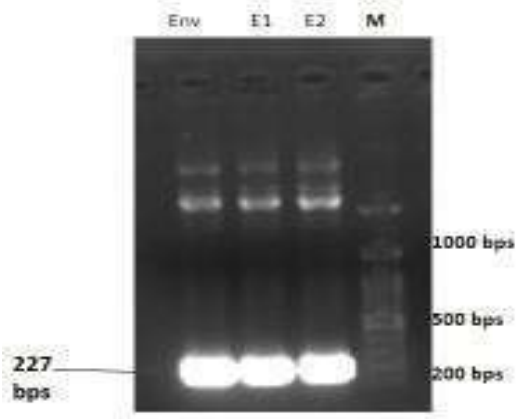
Preparation of Ni-NTA column and purification:

a) The protein purification column (25ml) was washed thrice with ddH_2O water and equilibrated by the extraction buffer.
b) The Ni-NTA beads stored at 4°C were removed and placed at room temperature for 5 minutes after shaking the beads thoroughly. Ethanol was removed from the beads and the beads were equilibrated by the extraction buffer after washing them with water thrice.
c) The supernatant (cell lysate) along with the beads (200µl) were allowed to incubate in a shaking condition overnight at 4°C, for efficient binding of the protein to the beads.
d) The beads were then loaded in the equilibrated column and were allowed to settle down.
e) The supernatant was allowed to flow through the beads and the flow through was collected.
f) The beads were washed with 10ml of Wash buffer (W1), (W2), (W3), (W4), (W5).
g) Collected 1ml of flow through from W1 and W5
h) Eluted the desired protein by taking 10 elution of 100µl elution buffer with 300mM of imidazole each.
i) Collected all the elations in a collection tube.
j) Pipetted out 15µl of each of the supernatants, flowed through, washed 1 and 5 and all the elutes (E1E5). 5µl of 4X loading dye was added to each and all the samples were boiled. The bacterial cell lysate was also prepared for loading.
The sample was loaded on a 12% SDS page, and after staining and destaining, the gel was checked for the desired protein in the transilluminator

Results:

PCR amplification of SARS-COV-2 Env from pcDNA3.1:

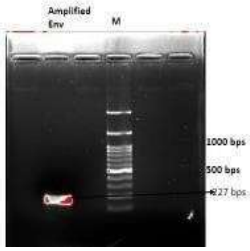
The amplification was done according to the procedure described above and the PCR product was run on 1% agarose gel. As we can observe in Figure (a), there is a 227bps band that could be seen on the gel which corresponds to the SARS Env.

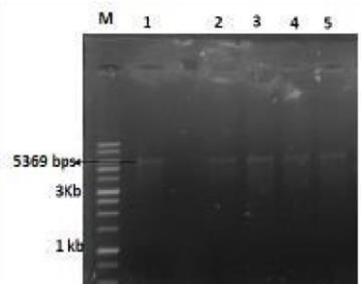


PCR AMPLIFICATION OF pcDNA SARS-CoV- 2
Env

Double digestion of SARS-CoV-2 Env amplicon pET28a vector with BamHI and EcoRI:

The PCR amplified product of SARS-CoV-2 Env was gel extracted and subjected to double digestion with BamHI and EcoRI. The Pet28a vector was also double-digested with the Above-Mentioned enzymes. There was an insert in the Pet28a vector previously, so when double digestion is done, if the insert was seen, then the double digestion of the Pet28a vector would be done properly. As we can observe in Figure (b), the digested SARS-COV-2 Env and pET28a vector can be seen at 227bps and 500bps respectively





(c) Double digestion of Pet28a vector

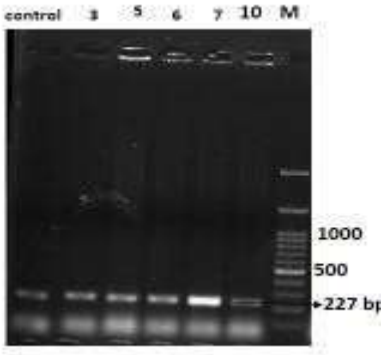
Ligation of the amplified SARS-CoV-2 Env inserts into the pET28a vector:

The digested SARS CoV-2 Env and Pet28a vector in the above gels were gel eluted and ligated by using the reaction mixture mentioned previously. The ligated mixture was transformed into DH5α cells. As we can observe in Figure (c), the colonies obtained were patched onto the kanamycin LB plate.

pET28a Env into DH5α cells

Colony PCR of pET28a Env:

The colonies obtained in the above plate were subjected to colony PCR. Among the colonies, the colony 7 was positive. This can be seen in Figure (d).



(d) Colony PCR Amplification of pET28a Env

Plasmid isolation and double digestion of pET28a Env:

The primary culture of the colony which was positive in the above colony

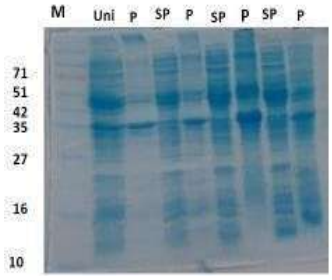
PCR and plasmid were isolated and double digestion was done using EcoRIand BamHI enzymes. The agarose gel in Figure (e) shows that there is a 227 insert coming out from the vector due to the double digestion which corresponds to the SARS-CoV2 Env insert.

Overexpression of pET28a Env in BL21 cells:

The plasmid was then transformed into BL21 cells, and the colonies obtained on the LB kanamycin plate after the transformation are shown in Figure (f). The colonies were picked, induction was given according to the procedure mentioned above and SDS gel was run. The SDS gel shown in Figure (g) showed the band at 12kDa which corresponds to the size of the SARSCoV-2 Env protein.

(f)pET28a Env-BL21 Cells

Patching of transformed pET28a Env-BL21 cells



(f)pET28a Env – Protein Expression

Purification of SARS-CoV-2 Env protein:

The protein was purified according to the protocol mentioned above in the materials and methods section. The SDS gel of the flowthrough, washes, and elution was run and this gel was stained and detained which is represented in the figure (h). A 12kDa band can be seen in this gel which shows purified protein corresponding to SARSCoV-2 Env.

purification of pET28a Env

Discussion and Conclusion:

Coronavirus E proteins are clearly important for virus assembly and egress, but their mechanistic role is still not understood. Genetic approaches have been used to examine the contributions of various conserved residues and domains to gain insight into the function of a protein.

Recent studies have shown that SARS E protein interacts with cellular proteins, some of which likely participate in the functional roles that the protein plays.

In the present study, the SARS-CoV-2 Env insert was isolated from the pcDNA3.1 vector. The insert was then subcloned into a bacterial expression vector such as the pET28a vector using Bam HI and EcoRI as digestion sites. The protein was then expressed at 1mM IPTG induction at a temperature of 30˚c overnight and purified using a Ni-NTA affinity chromatography procedure.

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