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Anushka Verma
Student, Department of Biotechnology, School of Life Sciences, Central University of Rajasthan, Bandar Sindri, Ajmer, Rajasthan, India

Anjana Sharma
Research Scholar, Department of Biotechnology, School of Life Sciences, Central University of Rajasthan, Bandar Sindri, Ajmer, Rajasthan, India

Suman Tapryal
Assistant Professor, Department of Biotechnology, School of Life Sciences, Central University of Rajasthan, Bandar Sindri, Ajmer, Rajasthan, India

Corresponding Author:
Anushka Verma
Student, Department of Biotechnology, School of Life Sciences, Central University of Rajasthan, Bandar Sindri , Ajmer, Rajasthan, India

In vitro refolding after Purification of E2-CH1 protein at pH 6.5 using SP-Sepharose cation-exchange chromatography

Anushka Verma, Anjana Sharma and Suman Tapryal

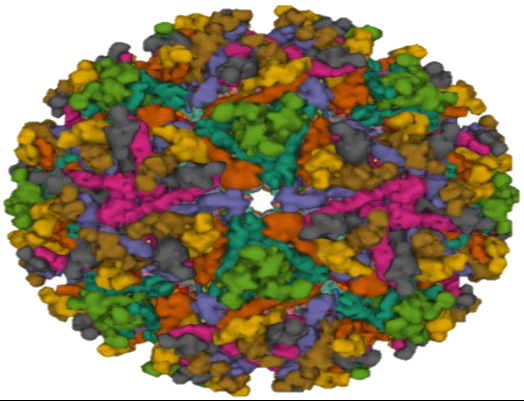
Abstract
Cases of chikungunya have been reported worldwide however the African, Asian, and the Indian subcontinents are the most affected areas. The virus once enters the host body, replicates in the midgut of a mosquito, and infect human until it dies. These mosquitoes mostly bite during the day and complete their life cycle in settled water bodies. Other than symptoms, serological tests like Elisa and RT-PCR are used as diagnostic tools. As there is no vaccination available for chikungunya now, the only treatment that exists is for relieving the symptoms. An attempt was tried to create a vaccine candidate for chikungunya by fusing the coat glycoprotein E2 with the constant region of the heavy chain of IgG. The competent *E-coli* cells transferred with E2 cloned pET28b vector and induced it with IPTG to express the protein. This protein was checked for solubility in pellet and supernatant and was further purified by phosphate buffer and refolded by SP Sepharose chromatography. This protein would be a potential vaccine candidate for chikungunya treatments.

Keywords: host body, replicates, pET28b

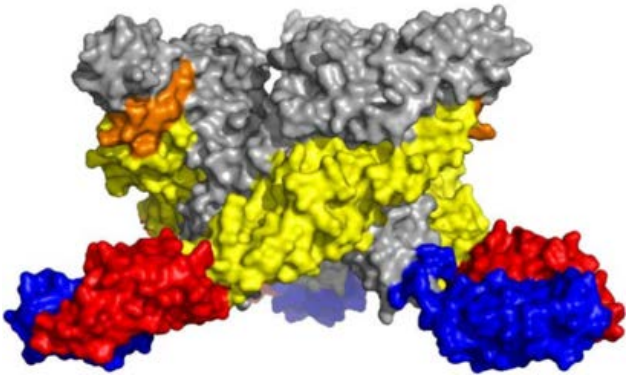
Introduction
The word '*Chikungunya*' is derived from the Makonde language of sub-Saharan Africa which translates to bending up (Thiboutot *et al*., 2010) [13]. Female Aedes mosquito spread chikungunya virus to human beings (van Duijl *et al*., 2015) [14]. Fever, joint pain, rashes, headache, nausea, etc. are the major symptoms observed in patients. Major affected continents have been noticed as Africa, Asia, and the Indian subcontinent however, sporadic outbreaks are commonly observed from time to time (Zeller *et al*., 2016) [15]. An Increase in number of Chikungunya cases in our country India has shown a significant increase since the year 2015 to 2019. Moreover, nearly 50 thousand cases were reported in the year 2019 which was nearly double to the number of cases had been reported in the year 2015. The human body develops IgM and IgG as anti-chikungunya antibodies are the infection (Zhang *et al*., 2019) [16]. Female Aedes mosquito spreads chikungunya virus that is positive-sense single-stranded RNA virus (de Caluwé *et al*., 2021) [4]. The size of the genome is about 11.8 kb in length composed of single-stranded RNA with a 5′ 7-methylguanosine cap and a 3′ poly-A tail (Kendall *et al*., 2019). Four structural glycoproteins (C, E1, E2, E3, and 6k), capsid and five non-structural proteins were coded by the genome. Glycoproteins E1 were involved in cell fusion and E2 was responsible for interaction with host cell receptors (Subudhi *et al*., 2018) [10]. The virion of CHIKV is composed of a lipid bilayer envelope connected to an icosahedral nucleocapsid shell which contains the RNA genome (Tanabe *et al*., 2018). Trimer of E1 and E2 heterodimer glycoproteins assembles an icosahedral lattice embedded in the viral envelope (Jin *et al*., 2019) [6]. The symptoms of *Chikungunya* infections are managed by non-steroidal anti-inflammatory drugs, anti-pyretic, and fluids as no legal vaccines or drugs are available to treat the patients (Gao *et al*., 2019) [5].

Method and Materials
The chikungunya virions are spherical, enveloped, and has a diameter of around 70 nm. The viral genome is present in the centre of a nucleocapsid core of diameter 35nm which is composed of capsid protein. The structural proteins of CHIKV, capsid, and three envelope glycoproteins are all expressed together and later separated by proteolysis. Glycoproteins include E1, E2, and E3 out of which E1 and E2 form a heterodimer, and a trimer of them create a spike (Castillo *et al*., 2018) [1].

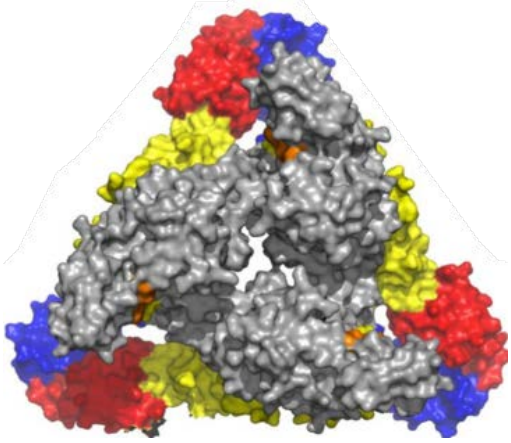
The surface of the envelope has around 80 spike proteins. The structural proteins cover the viral surface in the form of heterodimer spikes (Cho *et al*., 2008) [2]. The function of the spikes is to facilitate the attachment to cell surfaces and entry into the host cells. When pH is low E1 is responsible for membrane fusion during virus infection. The E2 envelope glycoprotein is a trans membrane glycoprotein that is responsible for receptor binding during the alpha virus cycle. E1 glycoprotein is 439 amino acids long with a conserved N-linked glycosylation site at 141 residue whereas E2 is 423 amino acids long with a glycosylation site at 263 and 345 residue (Madariaga *et al*., 2016) [8]. These glycosylation sites are conserved. A trans membrane helix of 26 amino acids and a 33 amino acids cytoplasmic domain are present at C-terminal. The interaction between cytoplasmic domain and nucleocapsid is important for the correct assembly and budding of progeny virus from the infected cells. The ectodomain of E2 has a size of 364 amino acids and consists of three immunoglobulin-fold domains termed A, B, and C, which are connected by a long β-ribbon. A mature chikungunya virion has 240 copies of E1 and E2 arranged as 80 trimeric spikes. Hydrogen bonds between these dimers stabilize the structure at neutral pH.



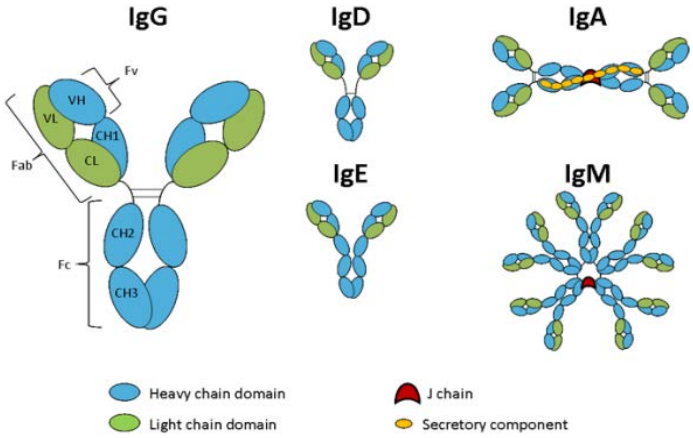
Electron cryo-microscopic image of Chikungunya virus



Surface model visualised using PyMOL of E1 (Side view)



Surface model visualised using PyMOL of E2 (Top view)



Diagrammatic representation of structures of different classes of antibodies

Antibodies are glycoproteins belonging to the immunoglobulin superfamily. They are proteins used by the body's immune system to fight against foreign pathogens (Schwartz and Albert, 2010). It is a Y-shaped molecule and the tip of antibody recognizes the antigen. The tip of the antibody is unique which is known as the paratope and it recognizes antigens epitope. The antibody recognizes the epitope and binds to it hence neutralizing the pathogen. There is a vast variety of antigens and each antibody binds to specific antigens. There are five classes of antibodies available in the human immune system *viz* IgA, IgD, IgE, IgM, and IgG (Sun *et al*., 2013) [11].

In the chikungunya virus-like particle, the structural proteins (E1, E2, and C) are expressed without any non-structural protein and genome to induce an immune response. The major antigenic determinants i.e. E2 and E1 are the vaccine candidates to be fused with IgG3 and IgM domains (Couderc and Lecuit, 2015) [3]. The appearance of neutralizing anti-CHIKV IgG3 antibodies was directly linked with virus clearance and long-term clinical protection. The study revealed that the naturally-acquired IgG response is dominated by IgG3 antibodies specific mostly for a single linear epitope. Most of the neutralizing epitopes were located on the E2 protein and a few of E1, therefore the heterodimer was trimmed to a stable size while carrying all the immunogenic epitopes. This design of vaccine will include E2 truncated protein fused to the N terminus of the heavy chain. E2 dimers are cloned in fusion with the IgM domain that showed the highest multi merization state. The protein E2-CH1 was a potential vaccine candidate.

Results and discussion

The discovery of inhibitory drug molecules that can interfere with virus attachment, endocytosis, and membrane fusion can prevent the development of persistent infection were highly valuable for treatment. Bacterial strain BL21(DE3) was the

most preferred host for expression. It carried a T7 RNA polymerase under control the of a lacUV5 promoter, allowed expression of the T7 RNA polymerase to be induced with IPTG, which made it a suitable host for induction and protein expression. E2CH1 gene was cloned in pET28b and was transformed into BL21 (DE3) strains. About 200µl aliquots of the competent cells were taken in an Eppendorf. Nearly 1µl of the plasmid DNA was added to it. After incubating for 30 minutes on ice it was given heat shock treatment by keeping cells in a water bath set at temperature of 42 °C for 90 seconds. Followed by an immediate incubation for 2 minutes on ice and then 800µl of autoclaved Luria Broth (LB) was added to it. The temperature of LB should be around 25 °C. The cell suspension was kept on an incubation shaker set at temperature of 30 °C for 1 hour at 150 rotations per minute. Lastly, 25µl out of the 1ml was plated onto an agar plate containing kanamycin antibiotic for the selection of transformants. The plates were kept in an incubator overnight at 37 °C. Good transformants show 60 to 100 colonies which can be used to prepare glycerol stocks.

Transformation of the cloned gene into the host

One drop of the *E-coli* glycerol stock (BL21) was used to inoculate 5ml LB broth which was incubated overnight at 150 RPM. 500µl of primary culture (1%) was used to inoculate 50 ml of LB broth in LAF. This was kept at 200 RPM at 37 °C for 2 hours until its O.D. reached 0.3-0.5. The secondary culture cells were cooled on ice and transferred to a pre-chilled sterile Oakridge tube under aseptic conditions. The cells were pelleted at 4500 RPM for 5 minutes at 4 °C. The supernatant was discarded and the pellet was resuspended in 25ml chilled 100 mM $CaCl_2$. The suspension was incubated on ice for 30 minutes and centrifuged at 4500 RPM for 5 minutes at 4 °C. Again, the pellet was resuspended in 3ml of 100mM $CaCl_2$ and 0.9 ml of glycerol was also added. The cells were resuspended on ice for 2-3 hours and finally store at -10 °C as 200µl aliquots.

Bacteria culturing

LB broth media was prepared by dissolving (25g/1000ml) in distilled water and autoclaved at 121 °C at 15 psi for 20 minutes. For the inoculation of primary culture, glycerol stocks were used. Antibiotics were added at 1000x dilution of the media and mixed thoroughly.

Induction and protein expression

1% of the primary culture was used to inoculate the secondary media containing antibiotics which were incubated in an incubator shaker at 200 RPM at 37 °C until its OD was reached 0.8. IPTG was used to trigger transcription and induce protein expression. A 1ml sample was taken every hour until 6 hours. At last, the culture was centrifuged at 4500 rpm for 15 minutes at room temperature. Both supernatant and pellet were kept for testing if the protein was soluble in the supernatant or insoluble in the pellet. Technique of SDS PAGE was used for confirmation of protein expression.

Sonication

Pellets were weighed and sonication buffer was added in the ratio of 1:9 along with PMSF (100x dilution of sonication buffer). After 20 minutes of incubation, the pellets were sonicated for a 3-minute cycle twice. After sonication, the suspension was centrifuged for 20 minutes at 6000 RPM at 4°C to obtain a compact pellet. The pellets were washed twice with washing buffer and Milli Q water. The pellets were resuspended in buffer and Milli Q water and pelleted by centrifugation at 4500 RPM for 30 minutes at 4 °C.

SDS PAGE and Sample preparation

After assembling the glass plates in the assembly unit 12% resolving gel (table-1) was prepared and poured in between the plates leaving appropriate space for stacking gel. Water was poured on top of it to prevent it from drying. When the resolving gel solidifies water was poured off and 5% stacking gel was poured and a comb is fixed into it (table-1). After a while, the glass plates were removed from the assembly unit and placed onto the buffer dam and the comb was removed. For sample preparation of pellets, they were weighed and appropriate autoclaved water and 6X protein loading dye was added. Nearly 50µl supernatant was aliquoted and 25µl of 6X protein loading dye was added into an Eppendorf (table-2). The samples were heated for 10 minutes at 95°C in a dry bath and loaded into the gels. The gel was run at 70 V until the dye reached the last point. The gel was carefully de-casted and kept in staining dye for 10-20 minutes and later kept in the distaining solution overnight.

IBs purification by SP Sepharose

After washing the pellets, purification was done. The pH of the buffer used was 6.5 and the buffer system was phosphate buffer. SP Sepharose was a cation exchange fast flow resin. Before purification, the SP Sepharose bed was prepared and regenerated. For Sepharose regeneration, 1M NaOH and 1M NaCl were used along with Milli Q water. Before and after NaOH and NaCl, Milli Q water was used for washing and centrifuged at 4000 RPM for 4 minutes at 4 °C. The pellets were solubilized in solubilization buffer (table-3) for 30-40 minutes on the rotator. For equilibration, solubilization buffer was added to the Sepharose and kept on the rocker for 15-20 minutes. Excess buffer was separated by centrifugation for 15 minutes at 6000 RPM at 4 °C. The solubilized pellets were added to Sepharose for binding and kept at the rocker for 1 hour. After binding the washes were given by wash buffer (table-3) for up to 7-bed volumes and centrifuged at 6000 RPM for 15 minutes at 4 °C. Elution buffer (table-4) was used for elution and eluted protein was collected in Eppendorf. SDS PAGE was used to confirm purification and the eluted samples were pooled for refolding.

In vitro refolding by dialysis

After purification, the pooled protein was diluted to a concentration of 1mg/ml by adding 10 mM β-mercaptoethanol and incubating for an hour at slow stirring. The dialysis bags were regenerated with 2% sodium bicarbonate. Purified protein was transferred to a dialysis bag and dialyzed against 800 ml of refolding buffer A (table-15) which was stirred continuously at 4°C. The concentration of urea was reduced by changing buffers (table-16 to 20). After 2M urea concentration buffer is supplemented with L-arginine and oxidized glutathione and urea is removed in further steps. After complete removal of urea, L-arginine and oxidized glutathione dialysis bag were transferred to last buffer G (table-21). All the dialysis steps were carried out at 4°C and stirred continuously. The refolded protein is removed from the dialysis bag and centrifuged to remove any residual aggregates and confirmed by 12% SDS PAGE.

Transformation of BL21 (DE3) strain of *E-Coli* using recombinant plasmid pET28b

The cloned gene for E2CH1 in pET28b vector was transformed in competent cells of BL21 and plated onto kanamycin agar plates to select the transformants. After 12-16 hours good transformed colonies were observed which were later used to inoculate the primary culture media.

Protein expression

The freshly obtained colonies carrying pET28-E2-CH1 plasmid were used to inoculate primary culture in kanamycin supplemented LB medium. The overnight grown primary culture was then used to inoculate fresh medium for secondary culture and culture was allowed to grow till OD600 of 0.8. At this OD600 culture was induced with 1 mM IPTG and thereafter samples of the culture were drawn every hour till the 6 hours post induction, wherein sample of 0 hour was used as un-induced control. The sixth hour sample was subjected to sonication and the supernatant and pellet fractions were prepared to assess the expression form of the heterologous protein using SDS-PAGE. As shown in Figure 1, a thick induced band of E2-CH1 was observed after one hour of induction of the culture and the expression levels were maintained till 6th hour post induction. Most of the recombinant protein co-precipitated with the pellet fraction indicating its accumulation in the inclusion bodies (IBs).

Purification

Large scale culture was set up to produce high amount of the protein, which was thereafter isolated from the soluble protein contaminants by simple centrifugation step. The IBs were washed with triton X100 as shown in Materials and Methods section and solubilized in PBS buffer containing 8 M at pH 6.8. The solubilized protein was thereafter subjected to cation exchange chromatography using SP Sepharose. After loading the protein sample, column was washed extensively and thereafter, the bound protein was eluted at 70 mM concentration of NaCl in 18 fractions of 2 ml volume each and analyzed on SDS-PAGE. As shown in Figure 2, purified and concentrated protein bands of E2-CH1 were observed in the eluted samples, however another lower-molecular-weight-contaminant-protein was apparently co-eluted with the protein of interest. The result shows that single step purification may not be enough for attaining high purification of the desired protein and another purification step may be required before in vitro refolding of the protein.

Refolding

Sometimes, purification may be achieved by refolding process as different requirements of refolding environments may force two proteins to refold at different pace leading to the efficient refolding of one of the proteins while other may precipitate in the process. Then, the two species of the proteins may be separated simply by centrifugation. Also, as the E2-CH1 protein was purified in the presence of 8M urea, the partially purified sample was immediately subjected to in vitro refolding. The purified protein was then subjected to in vitro refolding by a protocol wherein the concentrations of the denaturant and oxidizing agent were reduced slowly by dialyzing the protein solution against buffers carrying their continuously reducing concentrations, as described in Methods' section. Briefly, β- mercaptoethanol was added to the final concentration of 10 mM and the sample was incubated at 4°C constantly stirring. The concentration of urea was gradually decreased thereafter from 8M to 0M. Any of the protein aggregates were removed by centrifugation and supernatant was subjected to SDS PAGE. As shown in figure 3(a), after dialysis refolding, most of the protein was observed in the soluble fraction (supernatant) indicating the refolding of the heterologous protein. Thereafter, to analyze if E2-CH1 self-associate into a homodimer, the samples were prepared with 6X protein dye with β-mercaptoethanol (reducing condition) and without β-mercaptoethanol (nonreducing condition). As shown in figure 3 (b), the samples prepared with and without βmercaptoethanol, migrated at the same molecular wright band of the marker, indicating either E2-CH1 does not form a homodimer or the homodimer doesn't involve a disulphide bond formation. The protein's status with regard to its proper refolded state as well as its dimer formation, required further characterization using CD/FTIR spectroscopy and native page/conjugation analysis.

Protein estimation by Bradford assay

Bradford protein assay was used to find out the protein concentrations in samples. Coomassie Blue G250 was a red colour dye which turned into blue as it binded to protein. The absorbance of this dye has reached level up to maximum at 650nm and protein has an absorbance maximum of 590nm. Hence this dye was utilized to estimate protein concentration of 595nm. A stock solution of BSA was made and different concentrations were prepared (0.1mg/ml-1mg/ml) to plot a standard curve. Approximately 5µl of protein sample which was diluted in PBS was mixed with 250µl Bradford reagent in Elisa plate and absorbance was measured at 595nm. Finally, the total yield of the refolded sample was estimated by using Bradford assay (Figure 4) and the formula provided below and data values are depicted in Table 5.

Table 1: Stacking gel at 5% and 12% resolving gel

S. No.	5% stacking gel		12% Resolving gel		6X protein loading dye	
	Components	**Quantity**	**Components**	**Quantity**	**Components**	**Quantity**
1.	Distilled water	1.7ml	Distilled water	1.65ml	Tris base	4.56g
2.	Tris buffer (1.5M, pH-6.8)	315µl	Tris buffer (1.5M, pH-8.8)	1.25ml	Glycerol	60ml
3.	Acrylamide: bisacrylamide (30%)	415µl	Acrylamide: bisacrylamide (30%)	2ml	SDS	6g
4.	10% SDS	25µl	10% SDS	50µl	B-mercaptoethanol	6ml
5.	10% APS	25µl	10% APS	50µl	Bromophenol blue	0.006g
6.	TEMED	3µl	TEMED	2µl		
Volume		2.5ml		5ml		100ml

Table 2: Components with respective concentrations for solubilisation, wash and elution buffer

S. No.	Solubilisation buffer		Wash buffer		Elution buffer	
	Components	Concentrations	Components	Concentrations	Components	Concentrations
1.	Phosphate Buffer	10mM	Phosphate Buffer	10mM	Phosphate Buffer	10mM
2.	Urea	8M	Urea	8M	Urea	8M
			NaCl	5mM	NaCl	70mM

Table 3: Components and concentration for A, B and C buffers of the study

S. No.	Buffer A		Buffer B		Buffer C	
	Components	Concentrations	Components	Concentrations	Components	Concentrations
1.	Tris	50mM	Tris	50mM	Tris	50mM
2.	Urea	6M	Urea	4M	Urea	2M
3.	NaCl	50mM	NaCl	50mM	NaCl	50mM
4.	EDTA	1mM	EDTA	1mM	EDTA	1mM

Table 4: Components and concentration for D, E, F and G buffers of the study

S. No	Buffer D		Buffer E		Buffer F		Buffer G	
	Components	Concentrations	Components	Concentrations	Components	Concentrations	Components	Concentrations
1.	Tris	50mM	Tris	50mM	Tris	50mM	Tris-HCl	50mM
2.	Urea	1M	Urea	0.5M	Urea	0M	NaCl	50mM
3.	NaCl	50mM	NaCl	50mM	NaCl	50mM		
4.	EDTA	1mM	EDTA	1mM	EDTA	1mM		
5.	L-arginine	400mM	L-arginine	400mM	L-arginine	400mM		
6.	Oxidized glutathione	375µM	Oxidized glutathione	375µM	Oxidized glutathione	375µM		

Table 5: Bradford protein estimation

S. No	Sample BSA	BSA concentration (mg/ml)	Abs at 595nm	Concentration (mg/ml)
1.	0.1	5	0.292	0.827
2.	0.2	10	0.375	1.90
3.	0.3	15	0.482	3.2
4.	0.4	20	0.545	4.1
5.	0.5	25	0.607	4.9
6.	0.6	30	0.672	5.7
7.	0.7	35	0.787	7.2
8.	0.8	40	0.882	8.4
9	0.9	45	0.902	8.7
10.	1.0	50	0.989	9.8
11.	Blank	0	0.105	-
12.	Purified protein	-	0.353	1.61
13.	Refolded protein	-	0.347	1.53

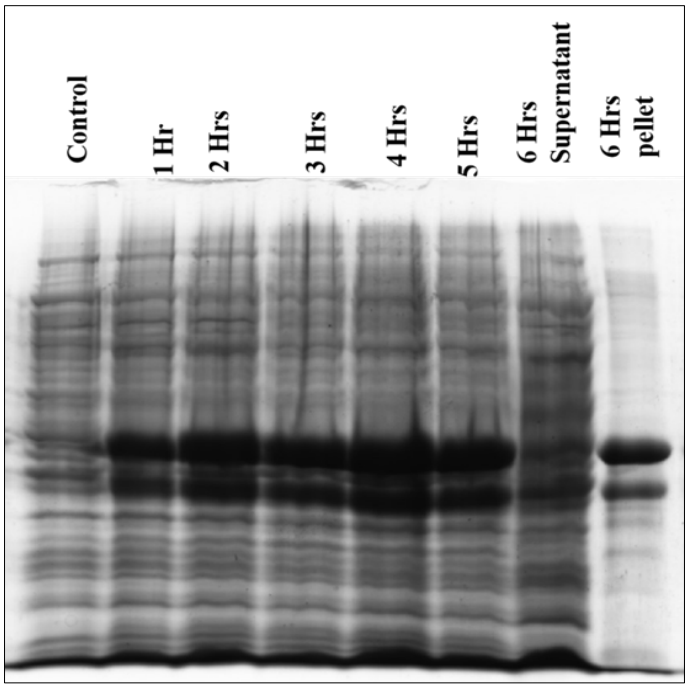


Fig 1: SDS-PAGE analysis of E2-CH1 protein expressed in BL2(DE3) cells

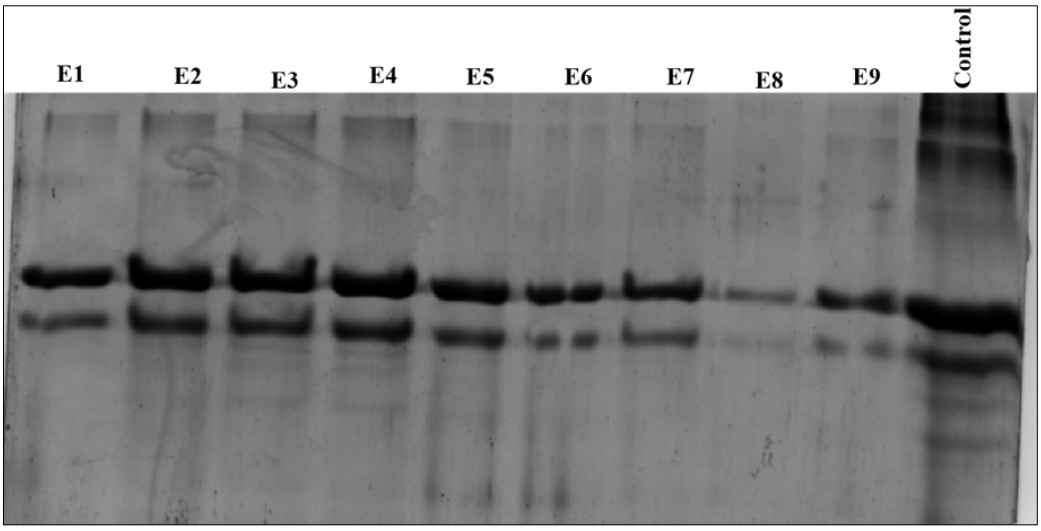


Fig 2(a): SDS-PAGE analysis of E-2-CH1samples purification using cation-exchange chromatography, samples E-1 to E-9 represents the ontinous samples eluted with 70mM NaCl; the crude washed IB sample was used as control

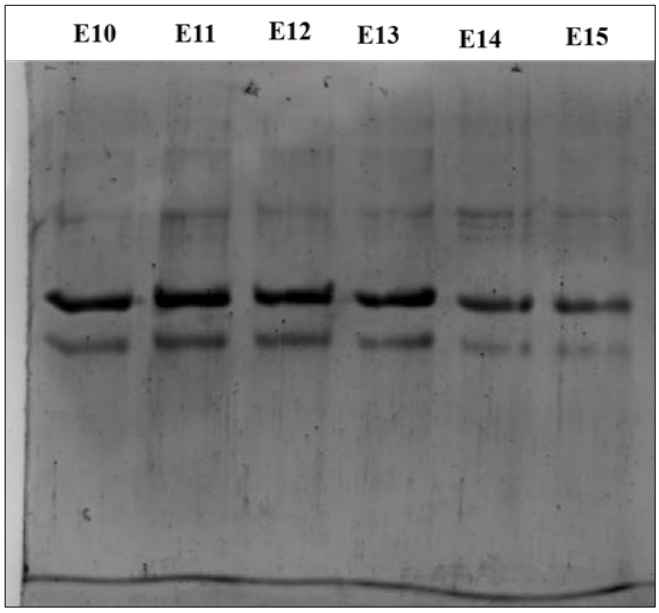


Fig 2(b): Eluted samples E10-E15

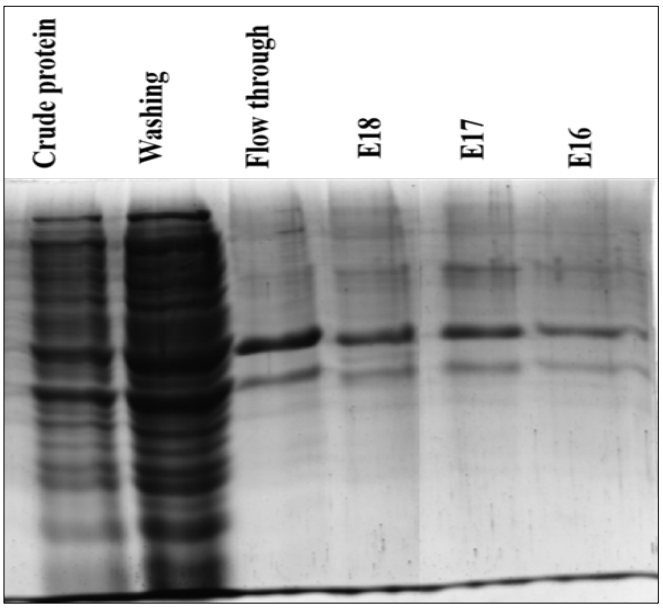


Fig 2 (c): Elusion samples E16-E18 and crude, washed and flow through samples of E2-CH11

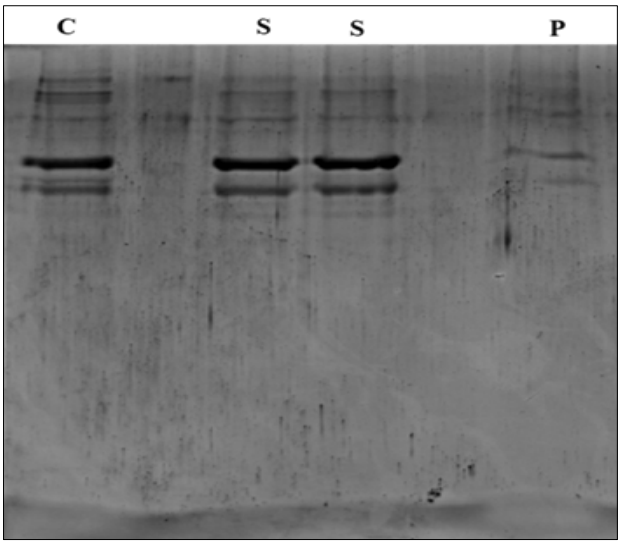


Fig 3(a): SDS-PAGE analysis of E-2-CH1 samples. The refolded sample was centrifuges at 9500 rpm for 20 minutes aand supernatant(s) and the pellet(p) samples were analysed. C denotes control purified-protein before refolding.

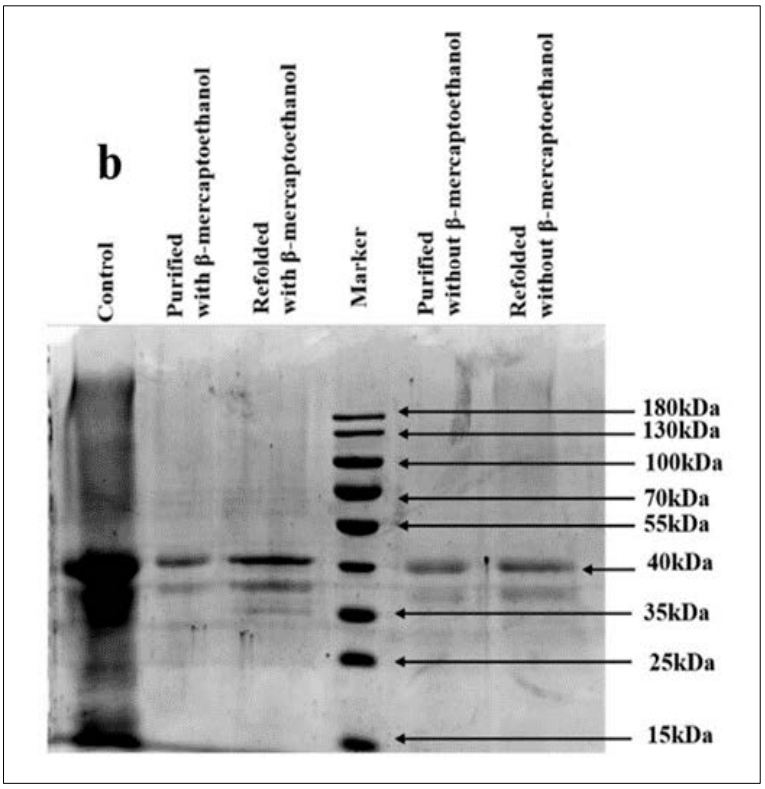


Fig 3(b): Analysis of E2-CH1 to form homodimer. The samples were prepared in presence and absence of β-mercaptoethanol

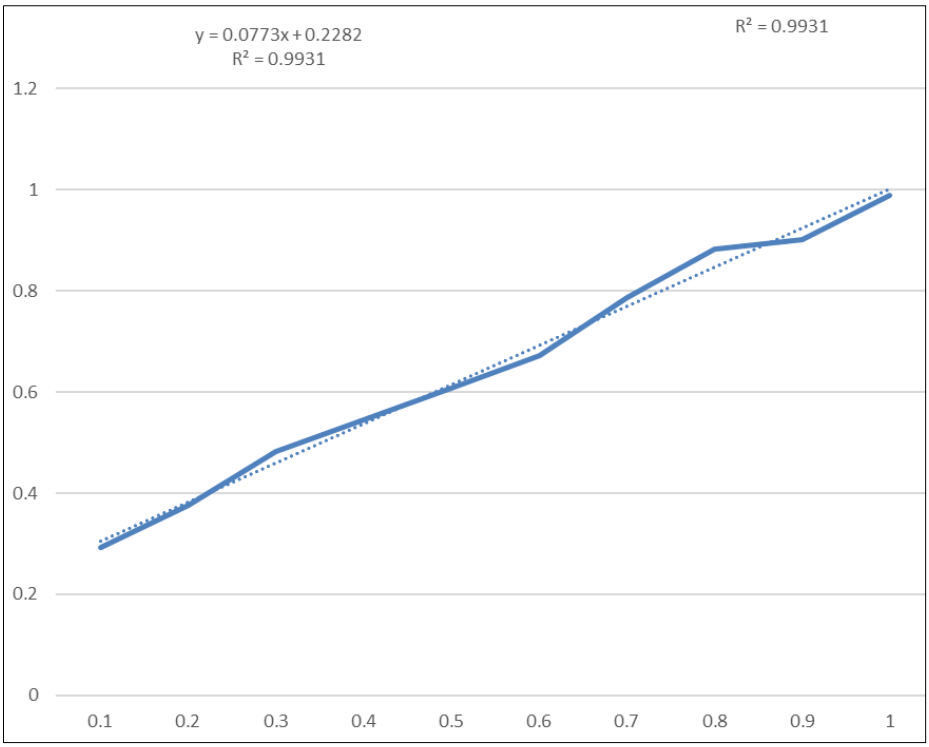


Fig 4: Bradford protein estimation graph Refolding yield = (final concentration after refolding / initial protein concentration before refolding) x 100 = (1.53/1.61) x 100 =95.03%

Conclusion

The need to develop vaccine against chikungunya is now more than ever. E2-CH1 forms protein in form of inclusion bodies and high yield of protein was obtained using 1mM IPTG as an inducer at 37°C for 6 hours. The proteins can be purified with SP sepharose chromatography, however for absolute purification, a follow up purification may be required. The purified protein sample was efficiently refolded, with a as high yield as 95%, using the protocol described in the current work. The purified and refolded vaccine candidate E2-CH1 may be combined with E1-CL counterpart protein to produce a potential vaccine candidate.

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References

1. Castillo-Macías A, Salinas-Carmona MC, Torres-López E. Immunology of viral infections with a high impact in Mexico: Dengue, Chikungunya, and Zika. Medicina Universitaria. Published online. 2018 Jan. doi:10.1016/j.rmu.2017.09.001
2. Cho B, Jeon BY, Kim J, *et al*. Expression and Evaluation of Chikungunya Virus E1 and E2 Envelope Proteins for Serodiagnosis of Chikungunya Virus Infection. Yonsei Medical Journal. 2008;49(5):828.
3. Couderc T, Lecuit M. Chikungunya virus pathogenesis: From bedside to bench. Antiviral Research. 2015;121:120-131.
4. de Caluwé L, Coppens S, Vereecken K, *et al*. The CD147 Protein Complex Is Involved in Entry of Chikungunya Virus and Related Alphaviruses in Human Cells. Frontiers in Microbiology. 2021, 12.
5. Gao S, Song S, Zhang L. Recent Progress in Vaccine Development Against Chikungunya Virus. Frontiers in Microbiology. 2019. 10. doi:10.3389/fmicb.2019.02881
6. Jin J, Simmons G. Antiviral Functions of Monoclonal Antibodies against Chikungunya Virus. Viruses. 2019;11(4):305.
7. Kendall C, Khalid H, Müller M. Structural and phenotypic analysis of Chikungunya virus RNA replication elements. Nucleic Acids Research. 2019;47(17):9296-9312.
8. Madariaga M, Ticona E, Resurrecion C. Chikungunya: bending over the Americas and the rest of the world. The Brazilian Journal of Infectious Diseases. 2016;20(1):91-98.
9. Schwartz O, Albert ML. Biology and pathogenesis of chikungunya virus. Nature Reviews Microbiology. 2010;8(7):491-500.
10. Subudhi B, Chattopadhyay S, Mishra P, Kumar A. Current Strategies for Inhibition of Chikungunya Infection. Viruses. 2018;10(5):235.
11. Sun S, Xiang Y, Akahata W. Structural analyses at pseudo atomic resolution of Chikungunya virus and antibodies show mechanisms of neutralization. Elife. 2013, 2.
12. Tanabe ISB, Tanabe ELL, Santos EC. Cellular and Molecular Immune Response to Chikungunya Virus Infection. Frontiers in Cellular and Infection Microbiology. 2018, 8.
13. Thiboutot MM, Kannan S, Kawalekar OU, *et al*. Chikungunya: A Potentially Emerging Epidemic? PLoS Neglected Tropical Diseases. 2010;4(4):e623.
14. van Duijl-Richter M, Hoornweg T, Rodenhuis-Zybert I, Smit J. Early Events in Chikungunya Virus Infection—From Virus CellBinding to Membrane Fusion. Viruses. 2015;7(7):3647-3674.
15. Zeller H, van Bortel W, Sudre B. Chikungunya: Its History in Africa and Asia and Its Spread to New Regions in 2013–2014. Journal of Infectious Diseases. 2016;214(5):S436-S440.
16. Zhang YN, Deng CL, Li JQ, *et al*. Infectious Chikungunya Virus (CHIKV) with a Complete Capsid Deletion: A New Approach for a CHIKV Vaccine. Journal of Virology. 2019, 93(15).