Structure-function analysis of the core cytolysin domain of *Vibrio cholerae* cytolysin

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

Dilraj Singh

AUGUST 2013

Certificate of Examination

This is to certify that the dissertation titled '**Structure-function analysis of the core cytolysin domain of *Vibrio cholerae* cytolysin**', submitted by Mr Dilraj Singh (Reg. No. MS08018) for the partial fulfilment of BSMS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Samarjit Bhattacharya Dr. Arunika Mukhopadhyay

Dr. Kausik chattopadhyay
(Supervisor)

DECLARATION

The work presented in the dissertation has been carried out by me under the guidance of Dr. Kausik Chattopadhyay at the Indian Institute of Science Education and Research (IISER) Mohali. The work has not been submitted in part or in full for a degree, a diploma or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the Bibliography.

Dilraj Singh
Dated: August 8, 2013.

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Kausik Chattopadhyay
(Supervisor)

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Abstract

Vibrio cholerae cytolysin (VCC) is a membrane-damaging cytolytic protein toxin belonging to the family of β-barrel pore-forming protein toxins. It is secreted by *Vibrio cholerae*, the causative organism of severe diarrhoeal disease cholera. It is secreted by the pathogen in the form of ~79 kDa precursor molecule, Pro-VCC. Upon removal of the N-terminal Pro-domain, the toxin is converted into the mature form of VCC. In its mode of action, VCC is shown to form transmembrane oligomeric pores in the membrane lipid bilayer of the target eukaryotic cells. In this process, the central cytolysin domain of the VCC molecule contributes the pore-forming stem region that participates toward formation of the transmembrane β-barrel. Apart from the core cytolysin domain, VCC harbors two additional lectin-like domains, which are unique to this toxin: a β-Trefoil lectin-like domain and β-Prism lectin-like domain. Presence of the β-Prism lectin-like domains appears to facilitate the mode of action of the VCC protein, presumably via regulating the membrane-binding process of the toxin. It is, however, still not clear how the β-Trefoil domain contributes toward the VCC mode of action. In this context, the overall aim of our present study was to investigate whether the core cytolysin domain of the VCC protein could function in absence of the accessory lectin-like domains of the protein. In this direction, we generated a double-deletion construct of VCC lacking both the lectin-like domains of the protein. The recombinant protein was expressed and purified to homogeneity using the heterologous protein expression system in *Escherichia coli*. Intrinsic tryptophan fluorescence spectra of the mutant variant showed red-shifted tryptophan fluorescence emission maxima, as compared to that of the wild type VCC. Such tryptophan fluorescence profile of the truncated protein was consistent with the extent of surface exposure of the tryptophan residues present in the core cytolysin domain. Comparison of the thermal melting profiles showed that the mutant molecule mimicked wild type VCC in terms of overall stability of the tertiary structural elements. The truncated variant of VCC protein showed lack of any hemolytic activity against human erythrocytes, thus suggesting complete loss of functional membrane pore-forming activity in absence of the lectin-like domains. Our data suggested that the core cytolysin domain of VCC was able to fold by its own, but required the accessory lectin-like domains for functional channel forming property.

List of figures

Abbreviations used

VCC……*Vibrio cholerae* Cytolysin
PBS…….....Phosphate Buffer Saline
SDS-PAGE…….Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
NTA……Nitriolotriacetic acid
DNA……Deoxyribonucleic Acid
PFTs ……Pore-Forming Toxins
CT……Cholera Toxin
TCP…….Toxin Co-regulated Pilus
E. coli……*Escherichia coli*

Chapter 1
Introduction

Vibrio cholerae

Gram-negative bacteria *Vibrio cholerae* is the causative organism of severe diarrheal disease cholera. It has caused seven pandemic outbreaks. *V. cholerae* is a motile, non-spore-forming, curved, rod-shaped bacterium of length 1.4-2.6 μm, with a single polar flagellum. The major virulence factor of the organism is the cholera toxin (CT). *V. cholerae* pathogenesis also requires additional virulence factors that include toxin co-regulated pilus (TCP) and *V. cholerae* cytolysin (VCC).

V. cholerae was first isolated by an Italian anatomist Filippo Pacini in 1854 after that Robert Koch, worked for 30 years on this disease. The *V. cholerae* outbreaks are mainly caused by only O1 and O139 strains, whereas all the non-O-antigenic strains cause mild infections only. The O1 type strain consists of two biotypes, E1 Tor and classical. Differences in the sugar composition of the heat-stable surface somatic "O" antigen are the basis of the serological classification of *V. cholerae*. Currently this organism has been classified into 206 different serogroup (Shimada et al., 1994; Yamai et al., 1997).

V. cholerae has two circular chromosomes and total length of DNA sequence is ~4 million base pairs. Chromosome 1 contains 2,770 open reading frames (ORF's), and chromosome 2 has 1,115 ORF's**.**

V. cholerae comes in contact with human body through ingestion of contaminated food or water. The bacteria enter the intestine; colonize in the villi of intestinal cells, and releases cholera toxin. Cholera Toxin (CT) is an enterotoxin made up of five B-subunits that form a pore to fit one A-subunit. CT is encoded from filamentous phage gene, CTXφ. A phage gene is also responsible for another virulence factor of *V. cholerae*, the toxin co-regulated pilus (TCP), which acts as a receptor for CTXφ. The gene for CT and TCP are encoded by a ~40 kb long *Vibrio* pathogenicity island (VPI).

Pore-forming Toxins (PFTs)

Pore-forming toxins (PFTs) are produced by many pathogenic bacteria. They are potent cytolytic agents that act to kill the target host cells. They form pores on the membrane of the target cells, and disrupt the permeability barrier function, thus leading to cell death. This form of attack is widely used by many organisms like mammals, mushrooms and plants. They differ from each other in the mode of their primary interaction with the cell membrane and the mechanism of membrane pore formation process. Many PFTs form transmembrane oligomers on the host cell surface, a process that involves a series of structural and conformational changes, such as to expose or generate hydrophobic motifs which can penetrate into the core of the cell membranes.

In response to the damage caused by these PFTs, many host cells have evolved mechanisms to sense the damage, to mount defence responses, and to repair the membrane. These responses involve variety of signal-transduction pathways.

Classifications of PFTs

Pore-forming toxins can be classified into different classes depending on the structural features being used by the toxins, so as to insert into the membrane to form functional transmembrane channels.

- α-PFTs
- β-PFTs

1.1 α-Pore Forming Toxins

α-PFTs employ α-helical structural motifs to form a functional transmembrane pore; for example, Colicins produced by *Escherichia. coli*, Diphtheria toxin produced by *Corynebacterium diphtheria* etc. These toxins normally contain three domains: one domain helps the toxin to bind with its receptor, second to transport the toxic domain inside the cell, and thirdly a toxic domain that kills the cell. In the mode of action of α-PFTs, the first step is to bind with the membrane followed by the unfolding of the pore-forming domain which releases the hydrophobic helices that initiate insertion. It is observed that some specific acidic residues act as pH sensors in these toxins which disrupt the H-bonding at three conserved aspartate residues, causing the unfolding of the helix, thus in turn exposing a tryptophan residue which interacts with the membrane and triggers the insertion of pore forming domain.

1.3 β-Pore Forming Toxins

β-PFTs generate transmembrane pores using of β-strands. These PFTs are secreted as water soluble proteins by bacteria, and then convert into transmembrane oligomeric pores in contact with target cell membranes. For example Aerolysin (produced by *Aeromonas hydrophylla)*,α-toxin (produced by *Staphylococcos aureus)* and Vibrio cholerae cytolysin (produced by *Vibrio cholerae*). β-PFTs exist in two distinct conformational states (a) the water soluble monomer (b) transmembrane oligomeric assembly. The mode of action of β-PFTs involves interaction of the monomeric units with host target membrane followed by oligomerization to form a functionl transmembrane channel. The transmembrane channel is formed by the contribution of an amphipathic β-hairpin by each contributing monomer to a form a pore in the shape of a β-barrel, hence β-pore forming toxins.

Vibrio cholerae cytolysin

Vibrio cholerae cytolysin (VCC) is a prominent member of the β-PFT family. It is produced by most of the strains of *V. cholerae.* It kills a wide range of eukaryotic cells. It causes fluid accumulation in ligated rabbit ileal loop. It is transcribed from 2.2 kb *hly*A gene. It contains an N-terminal signal peptide, followed by a pro-domain, and then the mature part of the toxin. There is a 15 amino acid long linker

region present between the pro-region and the mature toxin; the linker region acts as the cleavage site for different proteases. The protein is produce as pre-pro-toxin, the signal peptide guide this toxin to the periplasm of the bacterial cell, where it is cleaved, and the protein is secreted as pro-VCC. Then the protein undergoes proteolytic processing, and generates the mature VCC, which is capable of forming transmembrane oligomeric pores in the target eukaryotic cells.

The water soluble monomeric form of the toxin (PDB ID: 1XEZ) contains four different domains:

(1) **N-Terminal pro-domain:** Pro-domain is required for the proper secretion of this toxin. It has been seen that this domain blocks the protomer–protomer interactions, and prevent oligomerization. So, it is very important to remove this pro-domain via proteolysis for the toxin to become active.

(2) **Cytolysin domain:** Cytolysin domain is the structural and functional core of this toxin. It plays role into binding the host cell membrane. It contains a pre-stem region which is composed of two β-strands; contribution of this pre-stem region from seven monomers leads to the formation of the transmembrane β-barrel pore.

(3) **β-trefoil domain:** It is a putative lectin-like domain. From the structure and sequencing comparison it has been speculated that this domain is similar to many plant toxins, having sugar-binding properties.

(4) **β-prism domain:** It is a 15 kda domain. This domain has the sugar binding properties. It also plays important roles in the mode of action of VCC.

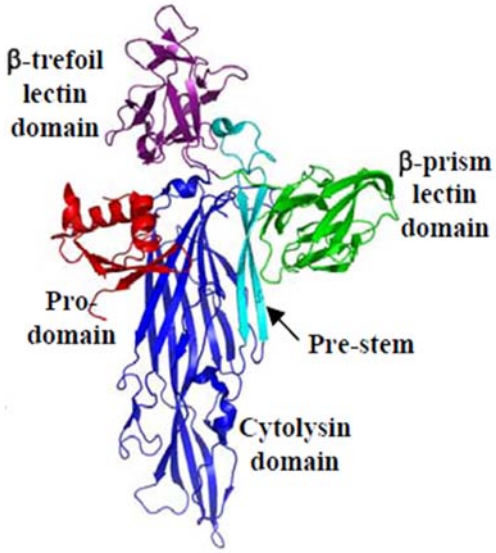

Figure 1. Structure of the monomeric Pro-VCC (PDB ID: 1XEZ)

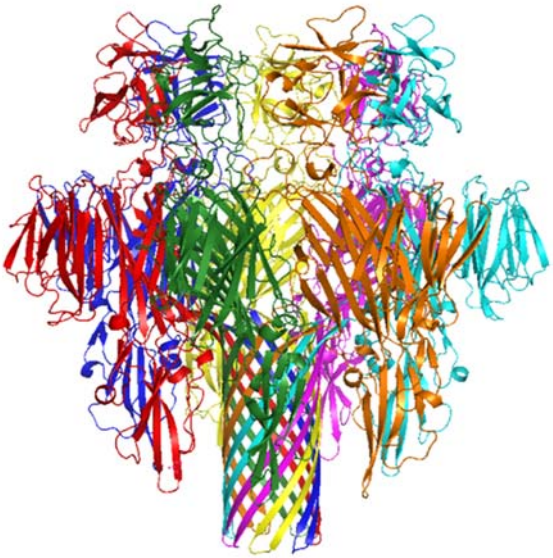

Figure 2. Transmembrane oligomeric form of VCC (PDB ID: 3O44)

In the mode of action of VCC, first it undergoes proteolytic cleavage at the linker region between the pro-domain and mature part of the toxin. The mature toxin binds to its receptor present on the membrane of the target host cell, forms heptameric assembly, and then the pre-stem loops of all the protomers open up, and insert into the membrane, and form pores which allow small molecules, ions and water to pass through the membrane, thus causing the colloid-osmotic lysis of the host cells.

Specific objectives

Apart from the core cytolysin domain, VCC contains two lectin-like domains that are not commonly present in the archetypical β-PFTs. In order to understand their roles in VCC mode of action, we wanted to study the structure-function properties of the core cytolysin domain of VCC in isolation, lacking the two lectin-like domains. Therefore, in this direction we have generated a truncated construct of the VCC (VCC36) lacking the two C-terminal lectin-like domains, and compared its structural and functional properties with those of the full length wild type mature toxin (VCC65).

Materials and Methods

2.1 Molecular Biology Methods.

2.1.1 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a method uses a thermostable DNA polymerase enzyme to amplify a gene sequence of interest .It is necessary to know the base pair composition of the short region flanking the sequencing to be amplified in order to design the primers (short oligonucleotides complimentary to one of the strands of the flanking regions in 5′=>3′ direction). The nucleotide sequence encoding the VCC- del_trifoil & del_prism protein eliminating region of N-terminal signal peptide was PCR amplified with the gene specific primers using the pET14b vector harbouring gene of wild-type VCC. Primers were designed based on the VCC-Wild type sequence. Restriction sites for NdeI and BamH1 were incorporated in the forward and reverse primers; Reverse primers also introduced a stop codon at the end of protein coding sequence upstream to BamHI site.

Forward primer:
TTAGTAcatatgCCAAAACTCAATCGTTGC
Reverse primer:
ACAAGTggatccTTAGCGGCCACCCGTGAA

A standard PCR was performed in a final volume of 40 µl containing template DNA (10-100ng),1µl of each primer and platinum PCR Platinum Supermix (Invitrogen) .The reaction was run in a C1000 Touch Thermocycler (Bio-Rad).After an initial denaturation step (94°C,5min) 30 cycles of denaturation (94°C,30sec),primer annealing (56°C,30sec) and fragment extension (68°C,1min) were followed by final extension (68°C, 5 min) before the end of PCR.

Standard PCR reaction mix per 40µl of total volume

Reagents Volume (in µl)

PCR Supermix 37µl
Forward primer 1µl
Reverse primer 1µl
DNA template 1µl

2.1.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to analyze DNA according to its molecular weight/electro mobility. To analyze plasmids and PCR products samples were mixed with 1/6 vol. of 6 x loading dye (Fermentas) and run on a 1% (w/v) agarose. The gel was prepared with 1 x TAE buffer (40mM Tris, 1% (v/v) acetic acid, 1mM EDTA, pH 8.0), which was also used to run the gel at 90 V. For estimation of the size and amount of DNA in the applied sample, a marker (1kb plus ladder Invitrogen) with defined fragment sizes and DNA amounts was run in parallel. Bands were visualized by Gel Doc™ EZ Imager (Bio-Rad).

2.1.3 DNA extraction from agarose gel

To extract DNA from agarose gels for further experiments the desired band was quickly cut from the ethidiumbromide agarose gel under UV-light with a clean, sharp scalpel. The obtained gel slice was weighed and DNA was extracted using a gel extraction kit (Qiagen), following the manufacturer's instructions. DNA was eluted from the filter column with 50µl H_2O.

2.1.4 DNA restriction enzyme digestion and Ligation into pET14b Vector.

Restriction enzymes were used to generate and verify plasmid constructs as well as to digest PCR product for efficient ligation reaction. The desired amount of DNA, varying from 200ng to 10µg, was cut with the appropriate amount of restriction enzymes BamH1 and Nde1 (New England Biolabs).Double digestion of both PCR product and undigested pET14b(Novagen) was performed by incubation at 37 °C for 2-4 hours. After double digestion DNA was verified by agarose gel electrophoresis and DNA was purified using the QIAquick Gel Extraction Kit (Qiagen).

Vector backbones and the desired DNA fragments were ligated using T4-ligase (New England Biolabs).DNA concentration was measured with NanoDrop2000 Spectrophotometer (Thermo Scientific). For a ligation reaction 50ng double digested vector DNA was incubated with a threefold molar excess of insert at 25 °C for 20 min.

2.1.5 Preparation and transformation of chemically competent *E. coli* Top10.

The treatment of *E. coli* cells with Ca^{2+} and some other divalent or multivalent cations induces competence and makes these cells to take up DNA from the environment. With this method, the *E. coli* strain Top 10 (Invitrogen) was transformed with the ligation reaction of the pET14b vector with the amplified fragments. Only one copy of the re-ligated plasmid can be stably introduced into each cell and further multiplied during the growth of the bacteria. These transformants can be selected for using ampicillin as a selection marker because of the presence of the ampicillin resistance gene in the plasmid.

A tip with few cells from single Top 10 colony was transferred to a tube with 5ml of LB medium without antibiotic. The culture was incubated for 16h at 37°C shaking at 200rpm and then inoculated 2% of culture into 10ml of LB were the cells were further grown until the optical density of the culture reached OD(A600) 0,4 – 0,6. After cooling on ice for 10 min. cells were centrifuged down at 5000 rpm at 4°C for 10 min. and the pellet was gently re-suspended in 10ml ice cold 100mM $CaCl_2$. The suspension was left on ice for 5min. and centrifuged as described above. The pellet was gently re-suspended in 5ml 50mM $CaCl_2$ and left on ice for 45 min. After addition of 1ml 80% glycerol in 100Mm $CaCl_2$, aliquots of 100µl were transferred

into 1.5ml Eppendorf tubes with a pipette and frozen immediately in -80°C.The cells were kept at -80°C until use.

Before transformation, cells were thawed on ice and used immediately after thawing. 5-10µl of the ligation reaction were added into 100µl of the competent cells, mixed gently by ticking against the tube and left on ice for 15 min. The cells were exposed to a heat shock of 42°C for exactly 60 seconds and immediately kept in ice for 5 min and incubated at 37°C and 200 rpm for 45–60 min after addition of 1000µl of pre-warmed (37°C) LB medium. Culture ware centrifuge at 5000 rpm for 1min then suspension was spread on agar plates containing ampicillin (50µg/ml) and incubated for 16 h at 37°C.

2.1.6 Colony PCR

Bacteria originating from a single colony can be used directly as template for PCR because the initial denaturating heat disrupts the cell walls and make DNA accessible. The presence of the pET14b plasmid containing the insert of interest in the colonies growing on ampicillin plates was verified by PCR using the cells directly as a DNA template. Colony PCR was also used to detect cloned fragments in the entry vector pET14b after ligation reaction.

Each colony chosen for colony PCR was rubbed on bottom of 0.2ml tube for PCR reactions and remains of the cells inoculated to 5ml LB Media containing ampicillin with same pipette tip. The PCR reaction mix was set up as described in 2.1, except that the total volume was adjusted to 20µl without a DNA template. In each screen, the primers specific for the pET14b vector were used (T7fw and T7rc), sometimes in addition with one of the insert specific primers. The program for the PCR was the same as used before (section2.1). The amplified products were separated from the template DNA with 1% agar gel containing TAE buffer and visualized as described in agarose gel electrophoresis protocol (2.2). Positive colonies showed a fragment of the size combining the insert flanking regions and the insert size.

2.1.7 Plasmid DNA isolation and conformation of positive clones.

Plasmid DNA from *E.coli* was extracted from overnight cultures using the DNA extraction mini-prep kit (Qiagen) according to manufacturer instructions. Positive construct was confirmed by release of insert fragment by double digestion of plasmid DNA by BamH1 and Nde1 as the protocol described above in section 2.4.Positive clone further confirmed by DNA sequencing (MWG biotech Pvt Ltd Bangalore, India).

2.2. Protein biochemical methods

Structural and molecular biology studies of proteins require large quantities of purified proteins. Over the past several year protein expression systems (*Escherichia coli,* baculovirus-mediated insect cell, *Saccharomyces cerevisiae* and several mammalian based systems) have been developed for large-scale recombinant protein expression. Each of these systems has its strength and weakness (yield, proper folding, post-translational modifications, etc).

2.2.1 Protein expression in *Escherichia coli Origami B cells*

For the expression of protein, the pET14b plasmid harboring the VCC del-trefoil & delβ-prism domain gene was transformed into *E.coli* Origami ™ B(DE3) Competent Cells (Novagen) as described in above section 2.5. After transformation single colony was grown in 5 ml LB culture medium (Hi-Media) containing 50µg/ml of ampicillin until it reached an OD_{600} of 0.6 and protein expression was induced by the addition of 1mM IPTG for 3hr at 37 degree and 200rpm. After expression of protein at 37°C and 200rpm for 3hr, the cells were centrifuged at 5000×g and cell pellets checked for protein expression by SDS-PAGE.

2.2.2 Protein purification from *Escherichia coli Origami B cells*

For purification of protein E.Coli Origami B cells harboring the pET14b/VCC del-trefoil & delβ-prism domain were grown at 37^0 C with continue shaking condition in LB broth medium containing 50µg/ml ampicillin until OD_{600} reached 0.6. Culture was then induced by 1mM isopropylthiogalactoside (IPTG) and further for 3hr at the same condition. Bacterial cells were harvested by centrifugation at 4000rpm for 15min at 4^0C and re-suspended in a buffer containing 20mM sodium phosphate, 150mM sodium chloride (pH 7.0) containing bacterial protease inhibitor cocktail(Sigma). Ultrasonic disruption used for lysis the cells. Insoluble inclusion body separated by centrifugation at 12000rpm for 30min at 4^0C. The crude inclusion body washed thrice with 2% Triton X-100 and subsequently the inclusion body was solubilized in PBS containing 8M urea under denaturing condition by constant stirring at 25^0C.

Protein was further purified by passing through Ni-NTA agarose affinity chromatography resins (QIAGEN) following manufacture's protocol. Inclusion body was adjusted with 20mM imidazole passed through the Ni-NTA agarose resin after that resin were washed with PBS containing 8M urea and 300 mM imidazole . Eluted protein was analyzed by SDS-PAGE and Coomassie staining.

Refolding of eluted protein was carried out by rapid dilution of the denatured protein(in 8M urea) into refolding buffer (PBS containing 2% glycerol) at room temperature with stirring for 20min. Refolding of protein was done by incubation at 4^0C for overnight into refolding buffer. After that refolding mixture was subjected to centrifugation at 12000rpm for 30min at 4^0C to separate out the insoluble aggregates

of the protein, the soluble fraction was collected and analyzed by SDS-PAGE for the presence of desired recombinant VCC protein.

Mature form of recombinant VCC was generated form Pro-VCC by treatment with trypsin (trypsin: protein weight ratio was 1:2000) for 10 min at room temperature. Maturation of Pro form of VCC remove Pro domain and protein get activated. Mature–VCC was further purified by passing through Q Sepharose Fast Flow resin (GE Healthcare).

2.2.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

Solutions used for SDS PAGE:

Stacking gel (3.8%)		**Separating gel (12%)**	
ddH_2O	3.25ml	ddH_2O	5ml
0.5M Tris pH 6.8	1.25ml	1.5 M Tris pH 8.8	3.75ml
10% SDS	50µl	10% SDS	50µl
30% acrylamide/0.8% bis-acrylamide	0.625ml	30% AA/0.8% BAA	6ml
10% ammonium persulfate (APS)	75µl	10% APS	75µl
TEMED	7.5µl	TEMED	7.5µl

Running buffer solution	**Coomassie staining solution**	**Destaining**
25 mM Tris pH 8.8	0.1% (w/v) Coomassie	10% (v/v) acetic acid
192 mM glycine	10% (v/v) acetic acid	25% (v/v) methanol
0.1% (w/v) SDS	25% (v/v) methanol	

To separate proteins according to their size denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was performed. Sodium dodecyl sulfate (SDS) is an anionic detergent that binds to most proteins in constant weight ratio. Proteins heated in presence of SDS denature into their primary polypeptides and gain an overall identical negative charge density. These polypeptides, migrating in an electric field towards the positive anode, can be then separated in a porous gel according to their size with smaller proteins migrating faster than the larger ones (Hames, 1998). For standard protein separation we used 10% acrylamide gels. Gels were poured using the BioRad device. Ammonium persulfate, which starts the polymerization (together with Tetramethylethylen-diamin [TEMED]) by radical formation, was added last just before pouring the gels. The stacking gel was poured on top of the separating gel when the latter had polymerized.

Purified protein with 1/6 volume of 6x SDS-PAGE Sample buffer (Fermentas), heated for 10 min. at 99°C. Protein sample (25µl) was loaded on the stacking gel. The low pH in the stacking gel and the comparatively high concentration of Acrylamide in resolving gel concentrates all proteins at a narrow

line at the beginning of the resolving gel after the voltage is applied. The gel was run for 2 h at 80V (BioRad PowerPac™). To visualize the separation efficiency the gel was stained for 1 h with Coomassie, swirling after that gel was destained overnight protein band was visualized with Gel Doc™EZ Imager (Bio-Rad).

2.2.4 Analysis of Hemolytic activity:

The liquid hemolysis assay with human blood cells was used to measure the hemolytic activity of the wild-type VCC and mutant VCC which lacking both lectin like domains. Human blood was washed with phosphate buffer saline (PBS) and diluted fivefold in PBS buffer. Both VCC proteins (800nM maximum concentration) were adder to 1000µl of blood cells and incubated at room temperature for 1hr. Samples were then centrifuged at 10000rpm at 4^0C for 10min. The absorbance of the supernatant at 415nm was measured to determine the released hemoglobin. The hemolytic activity of VCC-del-trefoil & delβ-prism domains was compared with that of recombinant wild-type VCC.

2.2.5 Fluorescence Measurements:

All fluorescence intensity measurements were performed using an Fluromax-4(Horiba scientific) spectrometer. The excitation wavelength and bandpass were 290 and 2.5 nm respectively. The emission spectra were observed form 310 nm to 400 nm.

Results and Discussion

Cloning, expression and purification of the Pro-VCC36

Nucleotide sequence encoding the Pro-VCC36 was cloned into expression vector pET14b (Figure 3), and expressed in *E.coli* Origami B cells (Figure 4). Both the Pro-VCC65 and Pro-VCC36 were purified under denaturing condition, from solubilised inclusion body in 8M urea, using Ni-NTA Agarose affinity chromatography. Upon elution from the Ni-NTA Agarose, the protein was dialysed against PBS containing 10% (v/v) glycerol.

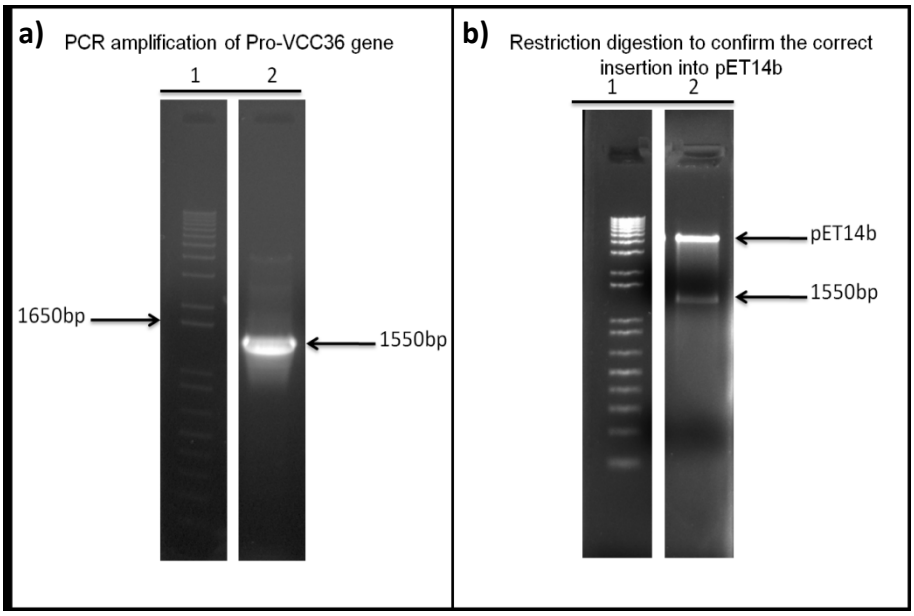

Figure 3. a) The shows the PCR amplification profile of Pro-VCC36 gene (b) and the restriction digestion with Nde1 and BamH1 to confirm the correct orientation in expression vector pET14b.

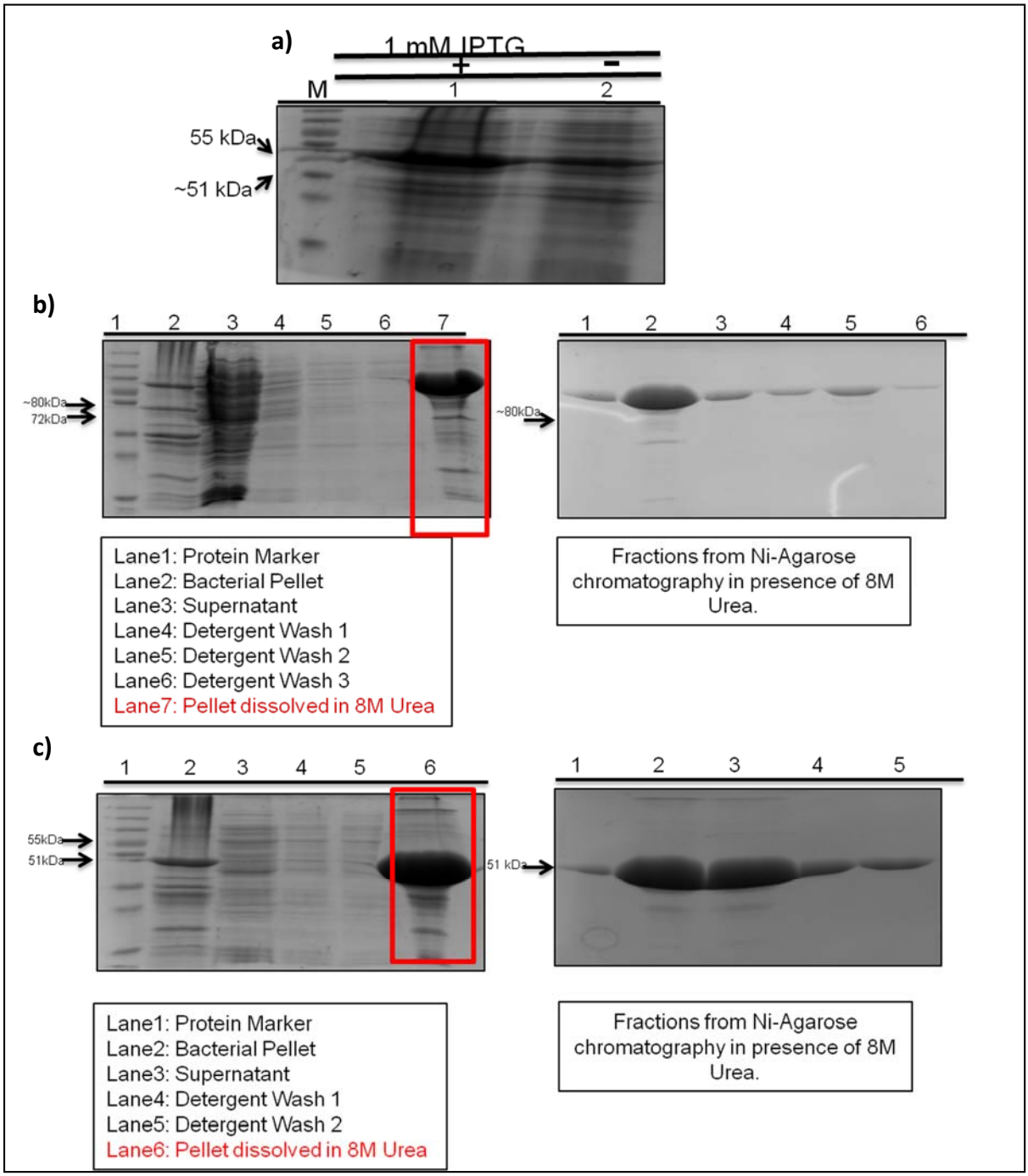

Figure 4. (a) Expression of Pro-VCC36 in *E.coli* Origami B cells, (b) SDS-Page profile of Ni-NTA-Agarose chromatography for Pro VCC65 and (c) SDS-Page profile of Ni-NTA-Agarose chromatography for Pro VCC36.

VCC36 displayed no hemolytic activity

After generation of the mature forms of the two variants, the proteins were assayed for hemolytic activity. Hemolytic activity was assayed in terms of hemoglobin released due to the lysis of erythrocytes. As expected, the wild type VCC65 showed 100% lysis at a concentration of 100nM, but the VCC36 did not show any hemolytic activity even at a higher concentration range of 750nM (Figure 5). This result showed that the core cytolysin domain of VCC was not able to form functional pores in the absence of the two lectin-like domains.

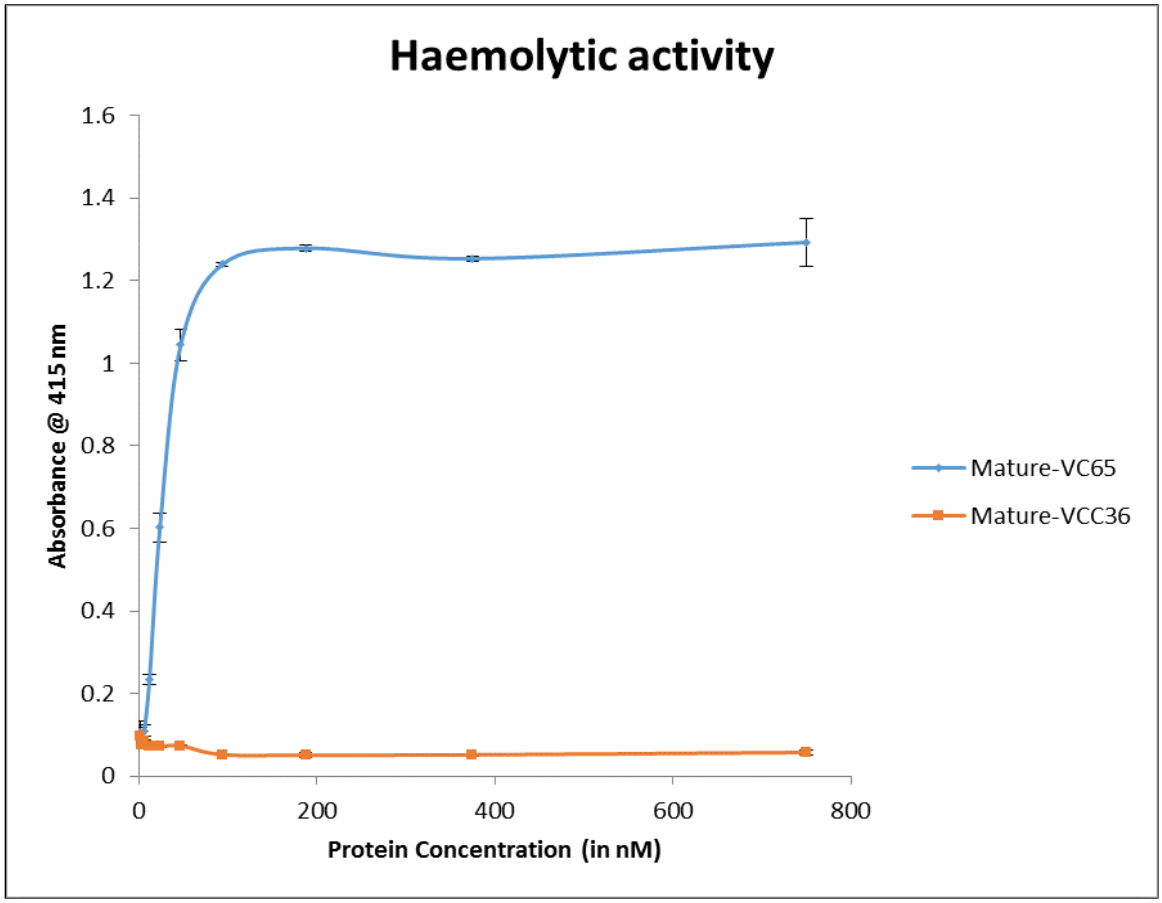

Figure 5: Haemolytic activity assay for Mature-VCC65 versus Mature- VCC36 using human erythrocytes.

Intrinsic tryptophan fluorescence shows similar tertiary structure for Mature-VCC65 and Mature VCC36

The mature forms of both the proteins were generated by proteolytic removal of the N-terminal Pro-domain (Figure 6). The Mature-VCC65 contains 11 tryptophan residues and Mature-VCC36 contains 7 tryptophan residues. Out of the 7 tryptophan residues in the core cytolysin domain of the VCC, i.e. the mature form of VCC36 molecule, 3 are partially exposed on the surface. The intrinsic tryptophan fluorescence spectra of VCC65 showed a tryptophan emission maximum at 340 nm upon excitation at 290 nm. The intrinsic tryptophan fluorescence spectra of VCC36 showed a red shifted emission maximum at ~343 nm (Figure 7). This could be explained by the fact that the VCC36 contains lesser number of tryptophan, out of which exposed tryptophan residues are more in number. Overall, our data suggested that the truncated form of VCC containing the core cytolysin domain maintained overall compact tertiary structure.

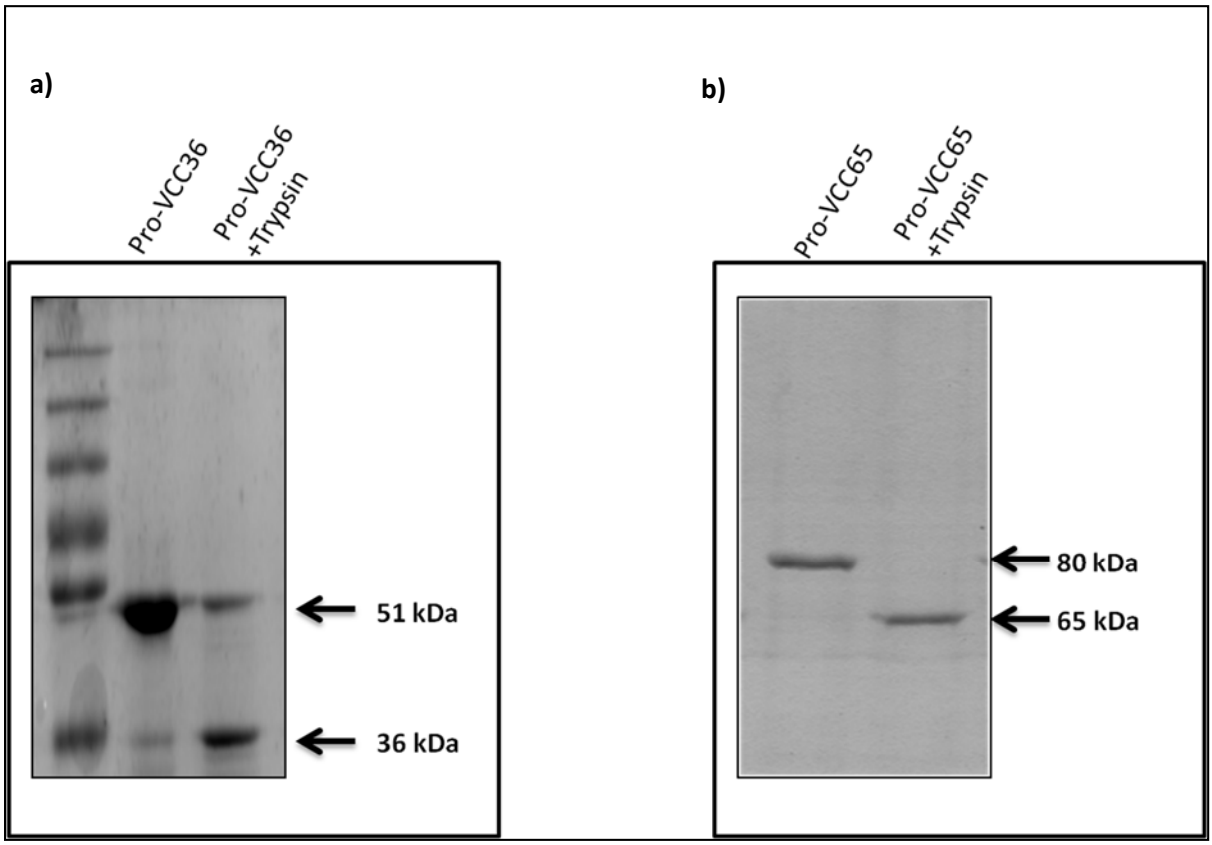

Figure 6. a) SDS-PAGE profile of Pro-VCC36 and Mature VCC36 and (b) Pro-VCC65 and Mature VCC65.

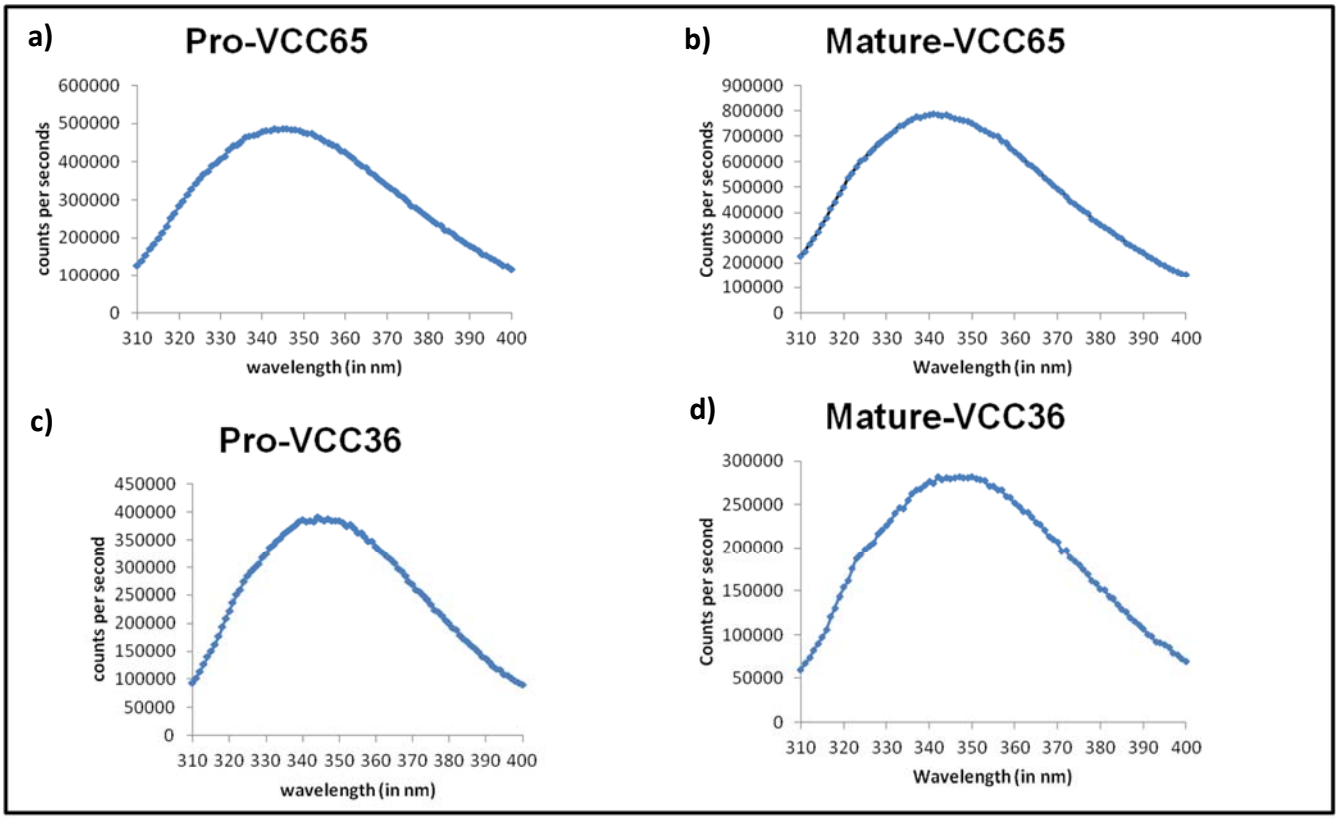

Figure 7: a) Intrinsic tryptophan fluorescence spectra for Pro-VCC65 (b) Mature VCC65, (c) Pro-VCC36 (d) Mature VCC36.

Thermal unfolding of VCC36 in comparison with VCC-65

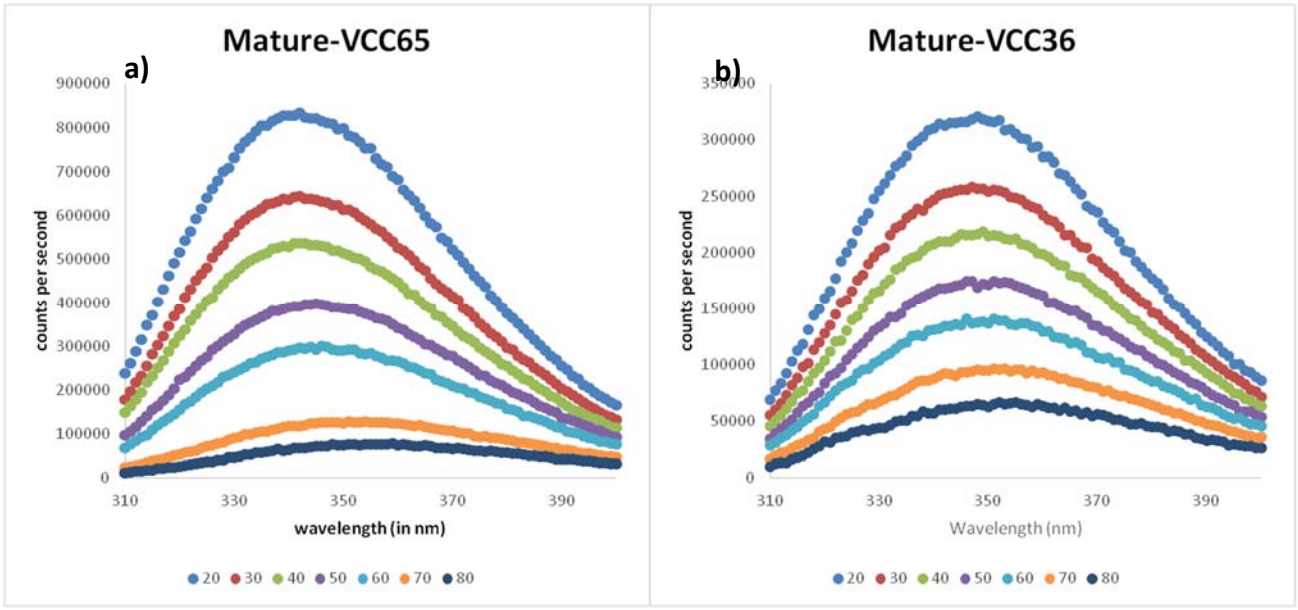

Figure 8: a) Thermal unfolding of Mature-VCC65 studied by Tryptophan Intrinsic Fluorescence (b) Thermal unfolding of Mature-VCC36 studied by Tryptophan Intrinsic Fluorescence.

To probe the stability of VCC36 in comparison with VCC65, we studied the thermal unfolding of both proteins using tryptophan intrinsic fluorescence. The spectra showed that both proteins started to unfold at the range of 60-70 °C, showing that the core cytolysin domain of VCC behaved similar to the full length protein in terms of thermal unfolding.

CONCLUSION

To study structure-function relationship of the core cytolysin domain of VCC, the cloned VCC36 variant was expressed in *E.coli* Origami B Cells and the protein was purified in the form of Pro-VCC36 from inclusion bodies. After removal of the N-terminal Pro-domain via proteolysis, the Pro-VCC36 was successfully converted to its mature form. In order to characterize this mature protein, VCC36 was compared with the full length wild type mature toxin (VCC65) in terms of its activity and structural stability. It was found that this protein lacks hemolytic activity implicating functional inactivity of the protein. Thermal unfolding and the intrinsic tryptophan fluorescence spectra suggested that the VCC36 mimicked wild type VCC65 in terms of overall tertiary structural stability. The present study shows that even though VCC36 behaves similar to that of VCC65 in terms of its structural elements but in absence of both lectin domains functionality of this protein is lost.

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