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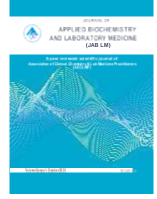

Original Research Article

Title: Heterologous expression of Salmonella typhi - Cytolethal distending toxin subunit B in E. coli and comparative viable bioactivity characterization.

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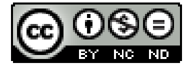

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ABSTRACT

Background: Cytolethal distending toxin subunit B (CdtB) is the active subunit of the *Salmonella typhi* holotoxin which is expressed intracellularly within host cells post-infection, and has been shown to induce typhoid-like symptoms. This subunit reportedly is a prospective candidate for typhoid diagnostics, and also finds application in cancer therapeutics. While, this protein is highly hydrophobic, and found to accumulate as aggregates (Inclusion Bodies) upon heterologous expression using a prokaryotic system, thus posing difficulties for scale-up. However, this issue can be resolved through optimization, to recover bioactive protein from protein-containing inclusionbodies.

Methodology: In this study, we have cloned a CdtB expressing gene from a clinical isolate of *S. typhi*, and also generated a truncated version of the same, devoid of some N-terminus hydrophobic patch. Proteins of both the forms were expressed using prokaryotic expression system and havebeen scrutinized fortheirviable functional activities through *in vitro* assays.

Results: Here, we provide a comparative account for the optimization of expression and purification of CdtB (wild type vs. truncated form), with the truncated version exhibiting a threefold increase in the overall yield and improved recovery post refolding. We have also demonstrated the characterization of their *in vitro* bioactivity, and found that both the CdtB forms retain their DNase and cytopathic activities.

Conclusion: Hence, through this study, we demonstrate a process for improved production of high quality and bioactive CdtB protein that can be utilized for downstream applications, like generation of cost-effective intermediates for *Salmonella* diagnostic and/or cancer therapeutics.

Running title: High-quality bioactive CdtB protein of S. typhi

Abbreviations:

CdtB: Cytolethal distending toxin subunit B; DNase: deoxyribonuclease; GRAVY, Grand average of hydrophobicity; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis

Introduction:

The typhoid toxin, an A_2B_5 type toxin, consists of three subunits pertussis-like toxin A (PltA), pentameric pertussis-like toxin B (PltB) and Cytolethal distending toxin subunit B(CdtB) **[1, 2]**. The holotoxin is assembled and secreted intracellularly in the host cells post infection **[2, 3]**. The systemic administration of the toxin is found to produce many symptoms of typhoid fever in mice, through the activity of its CdtB subunit **[4]**. The active component of the holotoxin, CdtB, is present in *S. typhi* and *S. paratyphi A* but is rarely found in other *Salmonella serovars* **[5]**. The CdtB subunit act as DNase because it shows sequence similarity with DNase I that inflicts DNAdamage and induces G_2/M cell-cycle arrest following autocrine and paracrine delivery of the toxin subunit into the target cells **[3, 2]**. Unlike other bacteria CDTs which are expressed efficiently under standard lab conditions, *S. typhi* CdtB expression is strongly induced upon host cell invasion and inhabitation of *Salmonella* containing vacuole (SCV) **[1]**. The specificity and secretory nature make CdtB a potential Typhoid diagnostic candidate **[6, 7]**, while its DNase property and the tendency to cause G_2/M arrest can be harnessed for development of potential anti- cancer modalities **[8-10]**.

CdtB is a 269 amino acid subunit protein of the typhoid toxin containing multiple interspersed hydrophobic patches, resulting in a high hydrophobic index. CdtB has three cysteine residues, two of which lie within an N-terminal hydrophobic patch, while the third being on C-terminal interacts with PltA **[4]**. Due to this scaled up production of CdtB protein is a challenge, which needs to be addressed for cost-effective generation of diagnostic or therapeutic intermediates, and biochemical studies. Recombinant protein production in aprokaryotic

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system, generally attributed to misfolding, aggregate formation and accumulation in inclusion bodies **[11]**. Additionally, proper refolding and recovery of bioactive protein from inclusion bodies (IB) is another challenge **[12, 13]**. Several interventions, such as host strains, protein solubilization, purification and even site-directed mutagenesis, etc., have beeninvestigated in thepast to improve proteinexpression **[14]**.

The study of the true potential of a candidate protein is governed by the availability of purified protein in high amounts with its activity intact. Thus in this study, we report the optimization of expression and purification of recombinant CdtB protein and its truncated form, ΔCdtB. We show the consequences of removal of N-terminal hydrophobic patches (1-29 amino acids) from the wild type CdtB to generate truncated ΔCdtB in order to obtain improved yield than the full- length protein with its functional activity intact. We also show the DNase activity and cytopathic effects of both the purified and refolded wild type and truncated CdtBproteins.

Material and methods:

Bacterial strains, Media and growth conditions

Enterobacterial strains *Salmonella enterica subsp. enterica serovar typhi, E. coli* strains *DH5-α*, BL21 (DE3)™ and Rosetta gami™ (DE3) obtained from Novagen-Merck, Darmstadt, Germany were used for protein expression. Expression vectors pPROEXHTb (Invitrogen, USA) and pET32a (+) (Novagen-Merck, Darmstadt, Germany) were used for cloning *S. typhi*, *CdtB* gene open reading frame (ORF). Other materials are enlisted in Supplementary Information (Supplementary Method, SM1).

CdtB and ΔCdtB amplification and expression plasmid construction

The genomic DNA from enterobacterial strain *Salmonella enterica* subsp. enterica serovar *typhi* **[15, 16]**, was isolated using genomic DNA isolation kit (Real Genomics) as per manufacturer's protocol. The CdtB gene amplification and cloning has been described earlier for the construction of pPROEXHTb-CdtB plasmid **[17]**. Other plasmid and clone constructions are described in Supplementary Method (SM2).The clones were confirmed by colony PCR, restriction digestion and sequencing.

Expression, Purification and Refolding of recombinant CdtB and ΔCdtB in E. coli

Protein expression optimization for the constructs pPROEXHTb-CdtB, pET32a(+)-CdtB, pPROEXHTb-ΔCdtB and pET32a(+)-ΔCdtB followed similar process as described previously **[17]**. Briefly, 1 L culture in fresh 2X TY media (1:100) was induced with 0.5 mM IPTG heterologous expression of proteins (CdtB and ΔCdtB). Target protein was confined majorly in inclusion bodies (IBs). Pure IBs were solubilized for purification of recombinant CdtB/ΔCdtB protein. The detailed process for purification and refolding of CdtB and/or ΔCdtB has been described in Supplementary method (SM3).

In vitro DNase activity

Supercoiled DNAnicking activity was determined with modification as described previously **[18]**. Briefly, 1 µg of supercoiled pGEM®-T DNA was incubated with varying amount of pure CdtB/ΔCdtB proteins in DNase buffer (25 mM HEPES, pH 7.0, 5 mM $MgCl_2$, 5 mM $CaCl_2$) at 37°C. The reaction mix was resolved on a 1% agarose gel post stopping with 10 mM EDTA, 10 mM EGTA, and individual bands were visualized with a BioRad GelDocsystem.

Cell Cytotoxicity and DNA Fragmentation assay

In vitro cellular cytotoxicity post CdtB or ΔCdtB treatment was performed on cultured HeLa cells (supplemental method SM5). For DNA fragmentation studies, HeLa cells (~2×10^4 cells) were grown on 0.01% Poly-D-Lysine coated 12-mm coverslips. Confluent (70-80%) cells were incubated with 3 and 1.5 µM CdtB or ΔCdtB for 24 h at 37°C in DMEM. Binding of proteins to HeLa cells were demonstrated by fluorescence microscopy (Supplementary-methods, SM6). The cells were PBS washed and fixed with 4% (w/v) paraformaldehyde (PFA). Coverslip were mounted using Fluoroshield™ mounting media with DAPI (Sigma, F6057) on a glass slide. The fluorescence microscopy was performed on an Olympus Laser Confocal Scanning Microscope (FV1000D) using a 40 × objective.

Cell Cycle Analysis

HeLa cells (5×10^5 per well) were seeded on 6 well culture plates (Nunc) and were treated with CdtB/ΔCdtB proteins (1.5 µM and 3 µM). Post intoxication, the cells were fixed and permeabilized by ethanol for 2 h and processed for flow cytometry. Homogenously 1 mL suspended 1 $\times 10^6$ cells were stained with 20 µg of propidium iodide (Molecular Probes) per mL in PBS. The stained cells were analysed by flow cytometrywith a FACScytometer (Beckton Dickinson)

Results:

Molecular cloning of CdtB and ΔCdtB

The amplicons from the isolated genomic DNA of *S. typhi* provided *CdtB* gene and its truncated form (i.e. Δ*CdtB*) of ~810 bp and ~732bp respectively. Further these were successfully cloned into different expression vectors to construct pPROEXHTb-CdtB, pET32a(+)-CdtB, pPROEXHTb-ΔCdtB and pET32a(+)-ΔCdtB plasmids (Fig.1A). Restriction mapping confirmed the positive clones with CdtB and ΔCdtB inserts. Additionally, sequencing analysis of positive clones confirmed 100% homology with the CdtB gene sequence of *S. typhi* (Accession No. AL513382.1. Removal of N-terminus hydrophobic region (AA 1-29) from the CdtB sequence reduced the Grand average of hydrophobicity (GRAVY) for the ΔCdtB from G=0.061 to G= -0.050 (Supplementary Fig. S1).

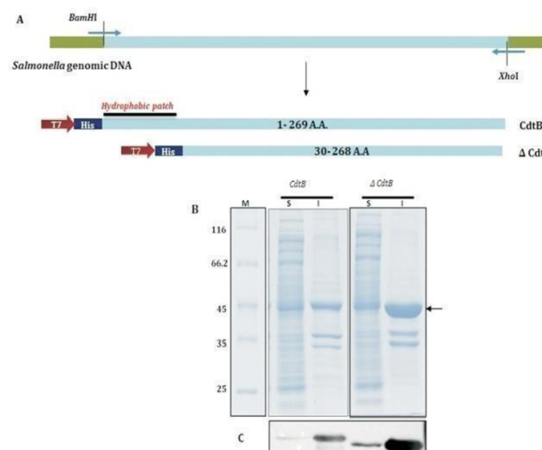

Figure 1.(A) Schematic diagram of cloning of gene encoding wild type and truncated (Δ) Cytolethal distending toxin subunit B (CdtB) in *E. coli* expression vectors pET32a+ and pPROEXHTb for expression of recombinant CdtB and ΔCdtB of *S. typhi.* **(B)** SDS-PAGE analysis of subcellular localization of CdtB and ΔCdtB. Cells transformed with pET32a+ constructs of CdtB and ΔCdtB were induced with 0.5 mM IPTG and grown for 15 h overnight at 18°C for protein expression. Cells were sonicated and separated into soluble and insoluble fraction. Protein samples were resolved on 15% Tris-glycine SDS-PAGE and stained with Coomassie brilliant blue. Lane M, Unstained protein MW marker (Thermo); Lanes S (soluble) and Lanes I (insoluble) fractions of CdtB and ΔCdtB (pET32a+). **(C)** Western blot analysis with anti-Histidine-tag antibody. Arrow indicates recombinant CdtB (~47 kDa) and Δ CdtB (~44 kDa) respectively.

Expression, Purification and Refolding of recombinant CdtB and ΔCdtB in E. coli

The constructs pET32a(+)-CdtB and pET32a(+)-ΔCdtB showed better expression in Rosetta gami™ strain, as ~47 kDa and ~44 kDa protein respectively, however, most of it was accumulated as intracellular aggregates. Albeit, the removal of the N-terminus hydrophobic patch (1-29; amino acid), improved the solubility of ΔCdtB protein and exhibited an enhanced overall expression as compared to the wild type CdtB protein (Fig. 1B, 1C). Pure IBs from pPROEXHTb- CdtB and pPROEXHTb-ΔCdtB transformed Rosetta gami™ strain contained ~85% CdtB (55 mg mL^{-1}) and ΔCdtB (60 mg mL^{-1}) expressed protein aggregates (Table 1, Fig. 2A, 2B).The step yield (%) post solubilization in 10 mL was 90% (5.4 mg mL^{-1}) in case of ΔCdtB and 83% (4.6 mgmL^{-1}) for full length CdtB(Table1).

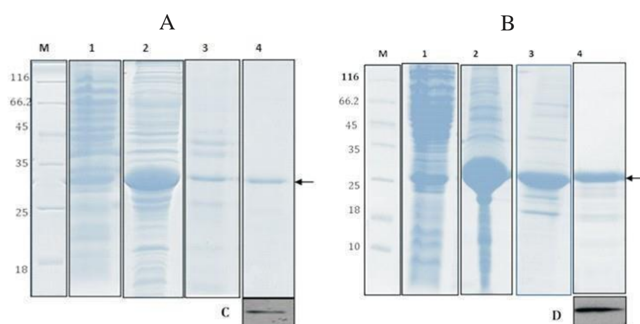

Figure 2. SDS-PAGE analysis of both expression and purification of recombinant CdtB and Δ CdtB from *E. coli* Inclusion bodies (IBs). Samples were analyzed on 15% Tris-glycine SDS-PAGE gel. Lane M; Unstained protein MW marker (Thermo), in **(A)** and **(B)** arrow indicates recombinant CdtB (~32 kDa) and Δ CdtB (~29 kDa) respectively, for both the pPROEXHTb constructs, Lane 1: total cell lysate; Lane 2: purified IBs; Lane 3: Solubilized IBs in buffer {50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 8 M Urea, 2 mM DTT and 0.1 % CHAPS}; Lane 4: purified protein after affinity chromatography. **(C)** and **(D)** Western blot analysis usinganti-His-tag monoclonal antibody.

Table 1. Purification and refolding efficiency of recombinant CdtB and ΔCdtB (pPROEXHTb constructs) proteins from Inclusion bodies (1 L Culture) of *E. coli* cells.

S. No	Parameters/ Fractions		CdtB		ΔCdtB		
			% Yield	*Amount (mg)*	*% Yield*	*Amount (mg)*	
1	**Inclusion bodies**		100	~55	100	~60	
2	**Solubilized protein**		83	~46	90	~54	
3	**AffinityPurified**		32	~15	79	~43	
4	**Refolded**	**Refolding Efficiency**	*in %*				*Buffer Compositions*
			60		80		50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 10 % Glycerol
			70		80		50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 10 % Glycerol, 0.4 M L-arginine
			30		40		50 mM Tris-HCl pH 8.0, 250 mM NaCl, 10 % Glycerol, 50 mM L-arginine, 50 mM Glutamic acid
5	**Pure bioactive protein**		**CdtB ~9 mg L^{-1}**		**ΔCdtB ~34 mg L^{-1}**		

Purified CdtB and ΔCdtB proteins (>90% purity as determined from SDS-PAGE band intensity) showed bands of ~32 kDa and ~29 kDa (for recombinant CdtB and ΔCdtB respectively) as confirmed by immunoblotting (Fig. 2A, 2B, 2C and 2D). Table-1 shows higher recovery of total ΔCdtB protein post affinity purification (43 mg), as compared to full length CdtB post purification (15 mg) from 1 L shake-flask culture. Conventional refolding by rapid removal of solubilizing agent, urea resulted in less than 10% recovery with aggregation (due to non- native or misfolded protein) from the total purified protein (data not shown). However, step-wise removal of urea supplemented with different additives, viz. 0.4 M of L-arginine hydrochloride, different buffers etc., considerably increased the % recovery of CdtB and ΔCdtB to 70% and 80 % respectively post refolding (Table 1).

Purified CdtB proteins exhibited functional DNase activity and in vitro cytopathic effects causing cell cycle arrested in cultured HeLa cells.

The DNase activity for purified and renatured recombinant CdtB and ΔCdtB was assessed on a supercoiled pGEM®-T plasmid DNA at different protein amount. Incubation with CdtB and/or ΔCdtB for 3 h at 37°C resulted in transformation of plasmid from supercoiled state to a linear state (Fig. 3-A, lanes 2, 3, 4, 5), and was completely hydrolysed after 24 h (data notshown).

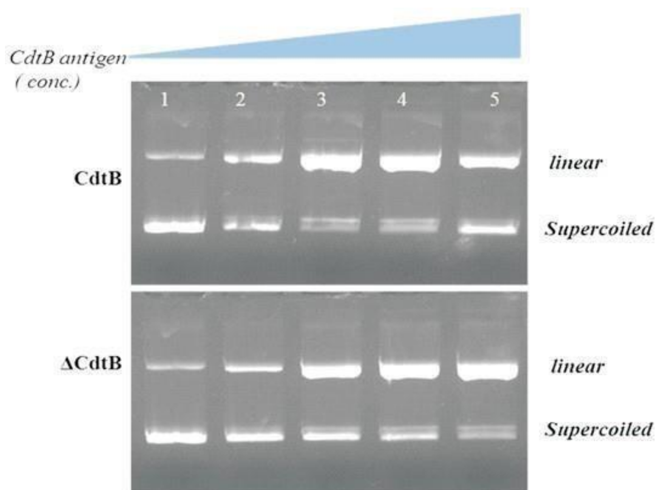

Figure 3-A. DNase activity of CdtB and ΔCdtB. Increasing amounts of purified protein was incubated with supercoiled pGEM®-T substrate. Lane 1, Buffer only. Lanes 2-5 indicate amounts of purified protein 0.25, 0.5,1, 2.0 µg respectively, of His-tagged CdtB and ΔCdtB. The agarose gel (1% agarose) was stained with ethidium bromide and visualized by UV transillumination.

Further we sought to determine whether purified target proteins exhibit cytopathic effects on cultured HeLa cells. Recombinant His-tagged CdtB proteins of *S. typhi* showed cell surface binding and internalization when HeLa cells cultured in presence of target proteins (Fig. S3). At 24 h after addition of the purified CdtB or ΔCdtB, morphological and nuclear change was detectable in majority of the cells, no major cell damage was observed in the buffer treated cells. The treated cell monolayers showed marked rounding and cellular distension, (D), Fig. 4A (inset), which corroborated previous studies. Nuclear staining with DAPI stain confirmed nuclear enlargement (E), fragmentation (F) and about 10% of the affected cells were found to be multinucleated (M) on visualization through Fluorescent microscopy (Fig. 3-B). We also observed significant reduction in cell survival (%), when the purified proteins were incubated with the HeLa cells in culture (Fig S2)

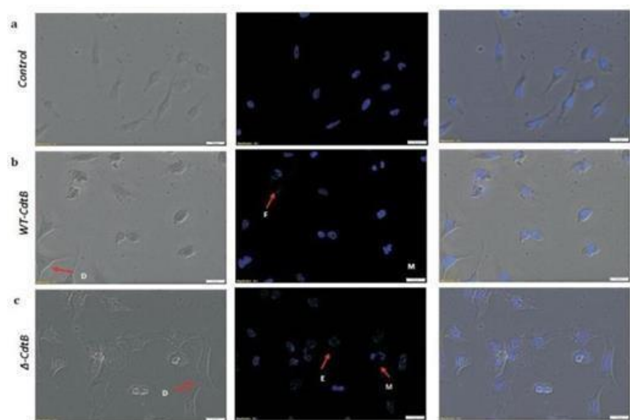

Figure 3-B. Cytopathic effect of *S. typhi* CdtB on HeLa cells. Compared to buffer treated cells **(a)**, cells treated with purified CdtB **(b)** or ΔCdtB **(c)** of *S. typhi* exhibited marked cytoplasmic distension, **D**; along with nuclear enlargement, **E**; multi-nucleation, **M**; and nuclear fragmentation, **F**. Magnification, 40x

Flow cytometry analysis for determination of the DNA content of treated HeLa cells was done to establish whether the morphologic changes observed in cells treated with recombinant CdtB and ΔCdtB of *S. typhi* were linked with cell cycle arrest. In untreated cell monolayers, the fraction of cells with G2-M stage as depicted byDNA content was consistently 8.2 % (Fig. 4B). In cells treated with recombinant 1.5 µM CdtB and ΔCdtB of *S. typhi,* an increase in the fraction of cells with DNA content was observed after 24 h treatment, showing to be 17 % and 22% respectively, suggesting G2-M arrest of the HeLa cells (Fig. 4B). Thus, recombinantly expressed proteins showed viable intrinsic functional characteristics.

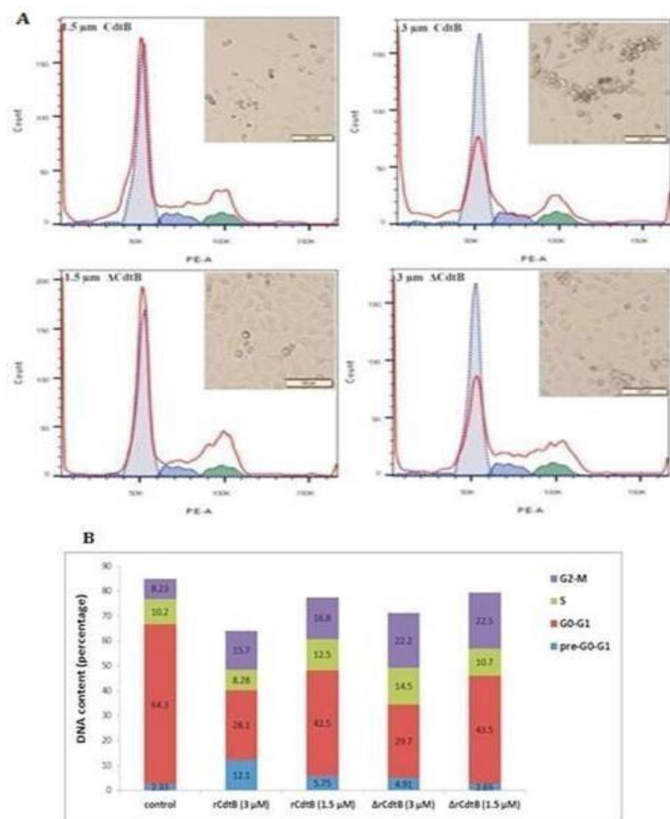

Figure 4. Effect of purified His-tagged CdtB and ΔCdtB proteins of *S. typhi* on cultured HeLa cells. **(A)** Purified CdtB proteins at concentrations 1.5 µM and 3µM (as indicated) were added to culture HeLa cells. At 24 h after addition of the proteins, the cells examined under bright field microscope (20x magnifications) for morphological changes or processed to measure DNA content by flow cytometry. Solid red line represents G, M, S phase of treated HeLa cells, dotted line represents G, M, S phase of untreated cells. (Scale bar = 100 µm; Images cropped for better visualization). **(B)** Histogram showing DNA content (%) in G2-M, S, and G0-G1 phases of cell cycle of untreated (control) and treated HeLa cells

Discussion

Recent reports have indicated CdtB of *S. typhi* to be a protein with immense diagnostic, prophylactic/therapeutic potential. Our group has also previously reported the utility of CdtB in accurate and efficient diagnosis of *Salmonella typhi* infections of humans **[17]**.

Furthermore, the evaluation of proteins like CdtB is limited by their expression in the inclusion bodies of *E. coli* cells **[19]** and low yields. Conventionally, solubility has been enhanced by; (i) mixing proteins with tags, (ii) co-expression with chaperones, (iii) using different hosts, but the effectiveness of such approaches is limited and the purification of proteins from inclusion bodies remains the only viable approach for certain proteins **[20]**.

Formation of protein aggregates as IBs in *E. coli* may be due to over-expression of a protein, reducing environment of cytosol (hampered disulphide bonds formation), and presence of hydrophobic amino acid in protein sequence **[21-24]**. The high hydrophobicity index exhibited by CdtB (G=0.061), which leads to greater tendency towards aggregation in inclusion bodies.

We followed the traditional approaches of expression with solubility promoting thioredoxin tag, redox host environment, low temperature, etc. independently as well as in combination with each other, but it did not seem to enhance the solubility of CdtB protein and >90% protein was expressed in the inclusion bodies itself. We therefore removed the N-terminal hydrophobic patch from the CdtB protein (truncation of the protein; ΔCdtB) with the aim of enhancing solubility. Earlier reports demonstrated removing hydrophobic patches improved the *in vivo* folding of scFv fragments **[25]** and, this strategy by analogy, can also be utilized to improve *in vivo* folding of other engineered protein domains. The optimization of solubility enhancement was carried out in conjunction with other traditional approaches and we found enhancement in protein solubility, but it was much lower than desired; therefore purification from inclusion bodies was essential in this case. Isolated pure IB from pPROEXHTb-CdtB/-ΔCdtB constructs, were although heterogeneous in consistency, and has highly enriched CdtB or ΔCdtB proteins in IB per litre of culture and yielded ~ 55 mg and ~ 60 mg protein respectively. Despite using harsh conditions (6 M Gu-HCl),reported earlier by others for IB solubilisation **[26]**, we could effectively recover approximately 83% and 90% of CdtB and ΔCdtB respectively, higher than that observed with mild to high solubilisation conditions tried with urea (4-8 M). The post-purification yield of ΔCdtB (~43 mg per L culture) under similar conditions was however found to be three-fold higher than CdtB (~15 mg per L culture) (Table 1). Protein refolding is often limited by improper misfolding and hence aggregation of the target protein. It therefore becomes imperative to optimize the refolding conditions to increase the refolding yield and improve the quality of the protein. We removed urea through step-dialysis and refolded the proteins using Tris-NaCl buffer supplemented with glycerol, L-arginine, equimolar mixture of L-arginine-glutamic acid as additives and found higher recovery with glycerol and L-arginine additives to the buffer **[27-29]**. The aggregated protein post dialysis was removed by centrifugation and the recovery (%) of refolded ΔCdtB in solution was found to be higher (80%) than wild type CdtB protein (60%). The removal of N- terminus residues from the ΔCdtB perhaps had a dual contribution in refolding; removal of a

large hydrophobic patch (reducing the G index to -0.050) and removal of all the cysteine residues, which might be responsible for lower yield of CdtB as aggregation of partially folded intermediates and non-native inter and intramolecular disulphide bond formation, can result in a significant decrease in final yields. Also, the Wild type CdtB protein aggregated beyond a final concentration of 300 μg mL^{-1} irrespective of the additive, while the ΔCdtB protein showed higher resistance towards aggregation at higher concentrations. This observation is consistent with earlier protein folding studies, which show that protein concentration is an important determinant in optimizing the yield of correctly folded protein **[30]**.

To ensure the proper refolding of CdtB and ΔCdtB and to explore the likelihood that the truncation of residues on the N-terminus would influence the activity of ΔCdtB, we analysed the *in vitro* activity of the purified proteins on epithelial cells (HeLa), and we found that both CdtB and ΔCdtB could function as a DNase, degrading supercoiled DNA, albeit with a bit compromised specific activity. Also, both the proteins could replicate *in vitro,* the biological activity of the CdtB protein on HeLa cells, i.e. cellular distension, nuclear enlargement and fragmentation with prolonged exposure to CdtB as observed with microscopy experiments, similar to previous reports **[31, 32]**. HeLa cells treated with the CdtB and ΔCdtB proteins also exhibited higher percentage of cells in the G_2/M phase thereby indicating arrest caused by the proteins, similar to the biological role of CdtB *in vivo.* It is notable that the residues crucial for the biological DNase activity of the CdtB and the residues comprising the nuclear localizing signal (NLS) for CdtB are present in both the truncated and full length recombinant CdtB proteins, and hence maintain their biological activity when properly folded. The efficiency of cellular delivery of the CdtB protein without any carrier proteins (PltA/PltB) or delivery agents has been shown to be low, but despite limitations in cytoplasmic delivery our recombinant proteins show significant biologicalactivity.

Thus, we could obtain sufficient amounts of refolded and bioactive CdtB (~9 mg L^{-1}) and ΔCdtB (~34 mg L^{-1}) with high purity (>95%) in an economical and convenient way by this method (Table 1). Moreover, achievement of a higher concentration of the purified protein also paves way for streamlined and cost effective production of the protein for evaluation as a therapeutic and/or diagnostic modality.

Conclusion:

Hence, a process for improved production of high quality bioactive CdtB protein was demonstrated, through this study. Although there have been numerous attempts for heterologous expression using different expression system, we have demonstrated the efficient and cost effective production process for CdtB proteins which can be utilized as intermediates for development of point of care *Salmonella* diagnostic tools, as one of their viable downstream applications. Moreover, recently we have evaluated the diagnostic potential for wild type CdtB proteins. However, therapeutic interventions through innovative improvisations of the bioactive recombinant CdtB protein for cancer need to be established.

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Conflict of interest:

The authors declare there are no direct and indirect conflicts of interest associated.

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