Understanding the Membrane Binding Mechanism of Thermostable Direct Hemolysin

Gunidhar Yengkhom

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

Department of Biological Sciences

Indian Institute of Science Education and Research Mohali

April 2015

Certificate of Examination

This is to certify that the dissertation titled "**Understanding the Membrane Binding Mechanism of Thermostable Direct Hemolysin**" submitted by **Mr. Gunidhar Yengkhom** (Reg. No. MS10067) or the partial fulfillment of BS-MS 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. Kausik Chattopadhyay (Supervisor) Dr. Arunika Mukhopadhyay Dr. Samarjit Bhattacharyya

Dated: April 24, 2015

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Kausik Chattopadhyay at the Indian Institute of Science Education and Research Mohali. This 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.

Gunidhar Yengkhom

(Candidate)

Dated: April 24, 2015

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)

ACKNOWLEDGEMENT

I am very grateful to Dr. Kausik Chattopadhyay, my supervisor for his useful advice, pertinent guidance and constant encouragement without which this project would not have been a success. I am very thankful to have an opportunity to work under him.

I would like to thank Miss Nidhi Kundu for her help and support in completing this project.

I would like to thank other lab members for their cooperation and support.

I would also like to thank IISER Mohali for providing me the necessary infrastructure and facilities to complete this project.

I am thankful to the faculty, staff and the students at IISER Mohali for maintaining a healthy research environment.

I am grateful to my family members for their encouragement and support.

Gunidhar Yengkhom

Lists of Figures

Lists of Table:

Abrreviation:

- PFT: Pore Forming Toxin
- TDH: Thermostable Direct Hemolysin
- WT: Wildtype
- LB: Luria Bertani
- RBC: Red Blood Cells
- IPTG: Isopropyl-β-d-thiogalactopyranoside
- PBS: Phosphate Buffer Saline
- PCR: Polymerase Chain Reaction
- Bp: Base pair
- SDS-PAGE: Sodium dodecyl sulphate – Polyacrylamide gel electrophoresis
- PC: Phosphatidylcholine

CONTENTS

INTRODUCTION

Every living cell is bounded by a lipid bilayer membrane known as plasma membrane which defines the integrity of cells. Plasma membrane isolates the cells from their external environment and acts as a permeability barrier. Bacteria have evolved to produce certain toxins that can breach the permeability barrier of the plasma membrane to gain access to intracellular environment of the cell or, to damage the target host cells. Pore-forming toxins (PFTs) are a class of toxins secreted by many organisms including bacteria, fungi, plants and even animals. PFTs constitute important virulence factors, and are most common bacterial protein toxins in a number of bacterial pathogens such as *Streptococcus pneumonia*, group A and B streptococci, *Staphylococcus aureus*, *Escherichia coli*, *Mycobacterium tuberculosis*, etc. (Gilbert R.J.C, 2002). Bacterial PFTs are widely studied to understand the mechanism of the toxin effect on the target cells. PFTs can damage membranes of host cells. Generally, PFTs are secreted as water-soluble monomers, and they oligomerise after binding to the target cell receptor to form water-filled pores in the host membrane (Gilbert R.J.C, 2002). They have the ability to form functional pores on plasma membrane of target cells. Formation of pores on the plasma membrane leads to colloid-osmotic cell lysis. Size of the pores formed by bacterial PFTs varies from small (0.5 to 5 nm) to large (20 to 100 nm) pores (Ferdinand C.O.L et al., 2013). Bacterial PFTs are mainly classified on the basis of the secondary structure with which the toxin gets incorporated into the plasma membrane of the target cells (Gilbert R.J.C, 2002). They are classified as 1) α-PFTs, which involve insertion of α-helices into the target plasma membrane. Some examples of α-Pore forming toxins are crystal (cry) toxins, cytolytic (cyt) toxins from *Bacillus thuringiensis*, Exotoxin A (ETA) from *Pseudomonas aeruginosa*, Cytolysin A (ClyA) from *Salmonella enteric*, etc. (Ferdinand

C.O.L et al., 2013). 2) β-PFTs which involve β-sheet structure like β-barrel to penetrate the toxin into the host cell plasma membrane. β-PFTs are most widely studied class of PFTs. Examples of β-PFTs are Panton-Valentine Leukocidin (PVL) form *Staphylococcus aureus,* Aerolysin from *Aeromonas hydrophila*, *Vibrio cholerae* cytolysin(VCC) from *Vibrio cholera,* etc. (Ferdinand C.O.L et al., 2013).

Thermostable Direct Hemolysin (TDH) is a PFT which is secreted from *Vibrio parahaemolyticus* (a gram-negative marine bacterium) (Honda T. et al., 1992). *V. parahaemolyticus* is one of the main causes of gastroenteritis associated with consumption of sea foods (Blake P.A et.al., 1980) Tsunesaburo Fujino discovered *V. parahaemolyticus* (at the time of discovery, it was named *Pasteurella parahaemolytica* later changed to *Vibrio parahaemolyticus* in 1963) after a seafood poisoning outbreak in 1950 (Shinoda S, 2011). *V. parahaemolyticus* is a member of Vibrio species from the family Vibrionaceae. *V. parahaemmolyticus* is free-swimming organism having a single polar flagellum (Shinoda S, 2011). These bacteria also have other virulence factors including adhesins, TDH-related hemolysin (TRH) and two type III secretion systems, T3SS1 and T3SS2 (Raghunath P., 2015). Thermostable Direct Hemolysin (TDH) and TDH-related hemolysin (TRH) are the two major virulence factors of this bacterium (Shirai H. et al, 1990). TDH and TRH share 70% sequence homology, and TRH has similar immunological functions as that of TDH (Kishishita M. et al., 1992). TDH has many biological activities such as hemolytic activity, cardiotoxicity and enterotoxicity. Type III secretion system (T3SS) from *V. parahaemolyticus* is also an important Toxin. They have two Non-redundant types, T3SS1 and T3SS2 (Park k. et al., 2004). T3SS1 is responsible for induction of autophagy, cytotoxity and mouse lethality, while T3SS2 plays role in enterotoxicity (Raghunath P., 2015).

Thermostable direct hemolysin was named because TDH is stable at 100 °C. TDH shows Arrhneius effect in which TDH becomes inactivated by heating at 60 °C but again reactivated by further heating to 90 °C (Miwatani T et al., 1972). TDH has the ability to lyse erythrocytes from many animals. TDH is secreted as a monomer consisting of 165 amino

acid residues (Tsunasawa S. et al., 1987). TDH monomer contains 10 β-strands and two helices, α1 and 3_{10} (Yanagihara I et al., 2010). It also contains one intra-molecular disulfide bond between cysteine residues at 151 position and 161 position of TDH monomer (Yanagihara I. et al., 2010). TDH monomers oligomerise to form tetramer in aqueous environment (Hamada D et al., 2007). After the formation of tetramer , TDH tetramer binds to plasma membrane and forms ~ 20 Å diameter functional pore which leads to colloid - osmtic lysis of the target cells (Yanagihara I et al., 2010).

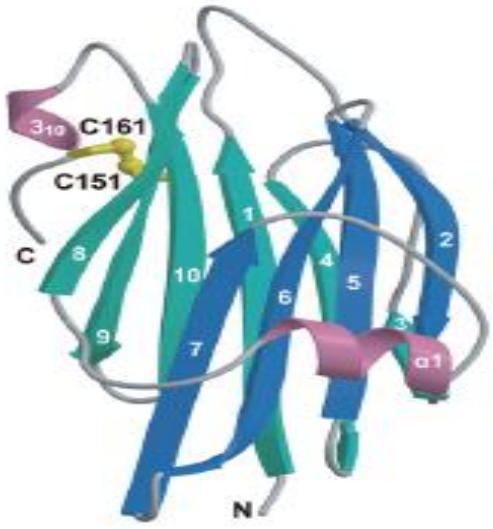


Fig 1: Ribbon representation of TDH monomer (PDB ID: 3A57) (Yanagihara I et al., 2010).

Unlike other PFTs, which generally form oligomer after binding of monomers to the plasma membrane, TDH has the unique feature of forming tetramer before binding to plasma membrane (Hamada D et al., 2007). TDH could recognize certain carbohydrates such as N-actetyl galactosamine, N-acetylglucosamine and galactose 1-3 N-acetyl galactosamine (Shekar M, Karunasagar I. 2013). There has been a report that TDH tetramer binds to phosphatidylcholine liposome (Yanagihara I et al., 2010). The binding mechanism of the toxin to the plasma membrane is still unclear.

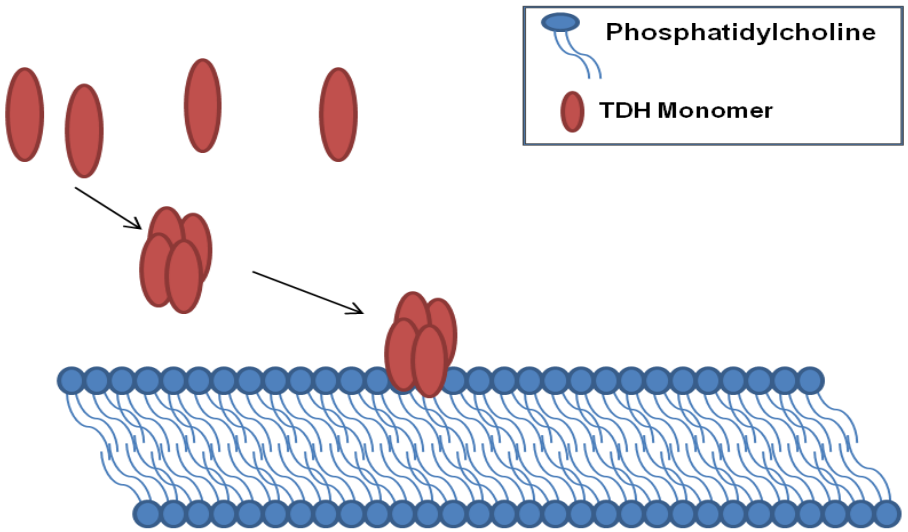


Fig 2: Formation of TDH tetramer and binding to Phosphatidylcholine.

There are no close sequence homologs of TDH. So, other similar PFTs have been searched, for which binding with phosphatidylcholine have already been known. Sticholysin II (StnII) is a PFT whose binding mechanism to phosphatidylcholine is well understood. StnII is secreted from *Stichodectyla helianthus* (Lucia G.O et al., 2011). StnII is a member of actinoporins proteins family. All the actinoporins act as PFT. Actinoporins reside in the nematocysts of sea anemones (Lucia G.O et al., 2011). When any prey comes in contact with their tentacles, these toxins are injected into the prey leading to noxious effect. Actinoporins are believed to function for predation, defense and digestion. Most of the actinoporins toxins share high sequence similarity (Lucia G.O et al., 2011). Sticholysin has two isotoxins known as StnI and StnII. StnI and StnII share about 91 % sequence similarity (Mancheno J.M et al., 2003). It is believed that Sticholysin exists as two isotoxins, to help the organism to prey a wide range of species. Most of the actinoporins are cysteineless. StnII monomer consists of 175 amino acids. StnII Phosphocholine (POC) binding site was established by solving X-ray structure of the complex (Mancheno J.M et al., 2003). StnII forms a pocket for binding to POC. Positive charge of choline moiety interacts with cation-π interactions with aromatic

rings of Tyr111 and Tyr135 and the phosphate group interacts with phenolic hydroxyl group of Tyr111 and Tyr135 (Lucia G.O et al., 2011). Binding of POC to StnII does not significantly change conformation of the toxin apart from a slight change in local side chain and backbone modification (Mancheno J.M et al., 2003).

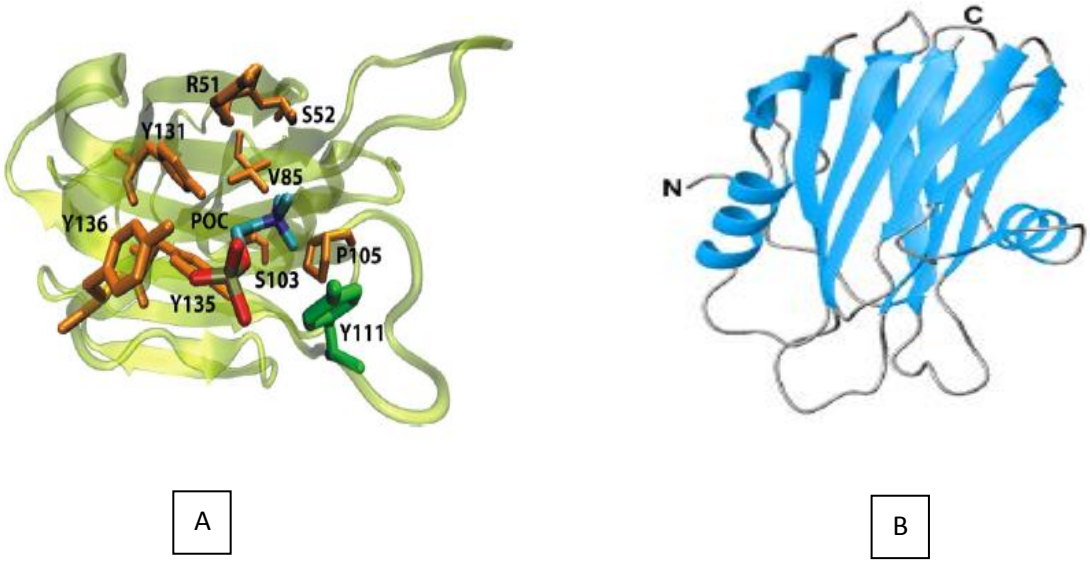


Fig 3: A) POC binding site in StnII (PDB ID: 1GWY1) and B) Ribbon representation of StnII (PDB ID: 1GWY) (Lucia G.O et al., 2011).

Tyr111 is reported to be critically important in binding with POC (Mancheno J.M et al., 2003). Tyr111 is 100 % conserved in all members of the actinoporins family (Lucia G.O et al., 2011).

To find, which amino acid residues in TDH are important in membrane binding, structures of TDH and StnII were superimposed to check if there was any amino acid residue in TDH which overlaps with Tyr111 in StnII. Tyr87 in TDH was found to be overlapping with Tyr111 in StnII. Variants of TDH were designed to check if Tyr87 in TDH is important in membrane binding.

Materials and Methods

1. Superimposition of Structures of TDH and StnII:

TDH and StnII structures were superimposed using Wincoot (based on secondary structure Matching, SSM) to examine if there was any amino acid residue in TDH overlapping with Tyr111 of StnII and that might be involved in binding with PC. PDB files of TDH (PDB ID: 3A57) and StnII (PDB ID: 1GWY) were retrieved from Protein Data Bank (http://www.rcsb.org/pdb)

2. Site-directed Mutagenesis by Overlapping Extension Polymerase Chain Reaction:

Recombinant variants of TDH, Y87A *tdh* and Y98F *tdh* were constructed using PCR-based method (overlapping extension polymerase chain reaction). Two rounds of PCR (polymerase chain reaction) cycles were done for each construct generation.

PCR program used:

1) 94°C for 5 minutes (for exensive denaturation),

2) 94°C for 30 seconds (for denaturation),

3) 54°C for 30 seconds (for annealing),

4) 68°C for 90 minutes (for extension),

5) go to step 2 and 30 X,

6) 68°C for 7 minutes and

7) 4°C till taken out.

For generation of Y87A *tdh*, we used WT TDH forward primer (Table 1), WT TDH reverse complementary primer (Table 1), Y87A TDH forward primer (Table 1) and Y87A TDH reverse complementary primer (Table 1). For first round of PCR cycles: A) 25 µl of 1X master mix (Invitrogen), 1.5 µl of WT TDH Forward primer (stock Conc. 10 µM), 1.5 µl of Y87A TDH reverse complementary primer (Stock conc. 10 µM), 1 µl of WT *tdh as* template

(60 ng/µl) and B) 25 µl of 1X master mix (Invitrogen), 1.5 µl of WT TDH reverse complementary primer (stock Conc. 10 µM), 1.5 µl of Y87A TDH forward primer (stock Conc. 10 µM), 1 µl of WT *tdh* template (60 ng/µl) were used. The samples, after the PCR cycles, were separated and checked by 1 % agarose gel electrophoresis. For second round of PCR, 40 µl of 1X master mix (Invitrogen), 1.5 µl of WT TDH forward primer (stock Conc. 10 µM), 1.5 µl of WT TDH reverse complementary primer (stock Conc. 10 µM) and 1 µl each from the products of first round of PCR cycles were used. Similar procedures were followed, as that of Y87A *tdh*, for making Y87F *tdh* in which Y87F TDH forward primer (Table 1) and Y87F TDH reverse complementary primer (Table 1) were used, instead of Y87A TDH forward primer and Y87 TDH reverse complementary primer respectively. The samples, after the final PCR cycles, were checked by 1 % agarose gel electrophoresis and the samples were extracted using gel extraction kit (QIAGEN)

Primers	Sequence (5'-3')
WT TDH forward primer	atattgCTCGAGatgTTTGAGCTTCCATCTGTCCC
WT TDH reverse complementary primer	AACATTggatccTTATTGTTGATGTTTACATTCAAAAAACGAT
Y87A TDH forward Primer	GCGGTGTCTGGCgccAAGCACGGTCATTCTGCTGTGTTCG
Y87A TDH reverse complementary primer	ATGACCGTGCTTggcGCCAGACACCGCTGCCATTGTATAG
Y87F TDH forward primer	GCGGTGTCTGGCtttAAGCACGGTCATTCTGCTGTGTTCG
Y87F TDH reverse complementary primer	ATGACCGTGCTTaaaGCCAGACACCGCTGCCATTGTATAG
T7 forward primer	TAATACGACTCACTATAGGG

TABLE 1: Details of Primers used.

3. Ligation Y87A *tdh* and Y87F *tdh* with a Plasmid Vector:

PCR product of Y87A *tdh*, Y87FA *tdh* and a cloning vector pET-14b (Novagen) were digested separately with both restriction enzymes XhoI (New England Biolabs) and BamHI (New England Biolabs) (incubated at 37 °C for 3 hours and then 10 minutes at 87 °C). Digested Y87A *tdh* and Y87F *tdh* were, separately, incubated for 1 hour at 24 °C with the digested pET-14b vector for ligation (Vector: Insert ratio was 1: 6, DNA was quantified using nanodrop spectrophotometer).

4. Cloning of Y87A *TDH* and Y87F *TDH* using an *Escherichia coli* strain:

4.1. Making of Competent Cells:

TOP10 *Escherichia coli* cells (Novagen) were made competent for transformation. Top 10 cells were incubated with shaking at 37 °C for overnight in 5 ml of 2.5 % Luria Bertaini (LB) (HIMEDIA) broth. 200 µl of the overnight grown culture was added into fresh 10 ml of 2.5 % LB media and incubated at 37 °C till optical density (OD) of the culture reached to 0.5 (OD was read at 600 nm in spectrophotometer). The culture was, then, centrifuged at 4000 rpm for 15 minutes at 4 °C. Supernatant was discarded and the pellet was resuspended in 10 ml of 0.1 M $CaCl_2$ (SIGMA). The suspension was kept on ice for 5 minutes followed by centrifugation at 4000 rpm for 5 minutes. The pellet was collected and resuspended it, in 5 ml of 50 mM $CaCl_2$. Again, the suspension was kept on ice for 45 minutes and centrifuged at 4000 rpm for 5 minutes. Supernatant was discarded and the pellet was mixed in 1ml of 85 % 0.1 M $CaCl_2$ and 15 % glycerol.

4.2 Transformation:

Competent TOP10 *E. coli* cells were transformed with PCR product of Y87A *tdh* and Y87F *tdh*, ligated with plasmid vector pET-14b (incubated in ice for 10 minutes, then, at 42 °C for 1 minute and again in ice for 5 minutes). 1ml of 2.5 % LB broth was added and incubated at 37 °C for 1 hour. It was, then, centrifuged at 5000 rpm for 2 minutes. Supernatant was discarded, but around 100 µl would remain at the bottom. The pellet was resuspended in the

remaining supernatant and the suspension was spreaded in agar plate containing 2.5 % LB, 1.5 % agar (HIMEDIA) and ampicillin (50 μg/ml) (HIMEDIA). Then, it was kept overnight in 37 ℃ to grow into colonies.

4.3. Colony PCR:

To ensure the gene of interest was ligated with the vector (pET-14b) which had been transformed in the competent E. coli TOP10 cells, we did colony PCR in which 10 colonies of the transformed TOP10 *E. coli* with Y87A *tdh* and Y87F *tdh* ligated with plasmid vector, pET-14b were taken, separately, in different PCR tubes and performed PCR (PCR protocol: 1) 94 ℃ for 5 minutes, 2) 94 ℃ for 30 seconds, 3) 54 ℃ for 30 seconds, 4) 68 ℃ for 90 minutes, 5) go to 2 and 30 X, 6) 68 ℃ for 7 minutes and 7) 4 ℃ till taken out) by making the colonies as template. We used T7 forward primer (Table 1), WT *tdh* reverse complementary primer (Table 1) and master mix (Genei). Presence of the gene of interest was checked in agarose (1 %) gel electrophoresis. From the 10 colonies, one colony each of Y87A *tdh* and Y87F *tdh* were choosen for further experiments.

The genes sequences of the constructs were confirmed by DNA sequencing.

5. Expression and Purification of WT TDH, Y87A TDH and Y87F TDH:

5.1. Small Scale Expression Check of WT TDH, Y87A TDH and Y87F TDH:

pET-14b vector ligated with our genes of interest were transformed in competent *E. coli* origami B cells (Novagen) (same procedures were followed as explain in (4.2)). A single colony of the competent *E. coli* origami B cells transformed with pET-14b (ligated with the genes of interest) was picked and added in 5 ml of 2.5 % LB broth containing ampicillin (50 μg/ml). When OD of the culture reached 0.5, IPTG (1 mM) was added. We used IPTG for inducing the expression of protein of interest. We checked the expression of our proteins on 12.5 % SDS-PAGE and Coomassie staining.

5.2 Proteins Extraction:

A culture of 100 ml (100 ml of autoclaved LB media, 100 µl of 50 mg/ml ampicillin and glycerol stock (1 ml) of competent *E. coli* origami B cells transformed with our genes of interest) was let to grow overnight at 37 °C. 20 ml of overnight grown seed culture was inoculated in 1 liters of 2.5 % LB broth containing ampicillin (50 µg/ml) and incubated at 37 °C with shaking at the rate of 180 rpm till OD (at 600 nm) reached 0.4 to 0.5. Then, incubating temperature was decreased to 30 °C and added IPTG (1 mM) in the culture allowing the proteins to be expressed and properly folded. After 3 hours of incubation at 30 °C, the culture was centrifuged (4000 rpm for 30 minutes at 4 °C) and the pellet was resuspended in PBS (20 mM sodium phosphate buffer and 150 mM NaCl) (pH- 7.4) followed by addition of protease inhibitor cocktail (Sigma-Aldrich). The cells were made to lyse by sonication (15 minutes; pulse on, for 30 seconds and pulse off, for 10 seconds; amplitude- 20). Then, it was centrifuged (12,000 rpm for 25 minutes at 4 °C) so that the protein remained in the supernatant. The supernatant containing the soluble protein was collected for purification.

5.3. Purification of WT TDH, Y87A TDH and Y87F TDH:

To the supernatant, containing the soluble proteins, imidazole (20 mM) (Himedia) was added and loaded onto Ni-NTA column. The column was, then, washed with 50 ml of 20 mM Imidazole to remove loosely bound unwanted proteins. The strongly bound protein of interest (containing His-tag) was eluted using 300 mM Imidazole.

After Ni-NTA chromatography, another round of purification was done using anion-exchange chromatography to ensure the purity of the protein. The samples eluted from Ni-NTA chromatography were diluted four times with 10 mM Tris-HCl buffer (pH- 8) (HIMEDIA) and loaded on Q-Sepharose column. It was washed with 20 ml of 200 mM NaCl and 10 mM Tris-HCl buffer (pH- 8) and protein was eluted in 500 mM NaCl; 10 mM Tris-HCl buffer (pH- 8).

The purity of the protein was checked on 12.5 % SDS-PAGE and Coomassie staining.

The purified protein contained his-tag from pET-14b vector at its N-terminal which needed to be cleaved. To cleave, the his-tag, protein samples were incubated with thrombin (1 units of thrombin for each 250 μg of protein) at 37 ℃ for 2 hours and then PMSF (Phenylmethane sulphonyl fluoride) (HIMEDIA) was added to inhibit the function of the thrombin. After thrombin treatment to cleave his-tag, we performed one round of anion-exchange chromatography (same procedures were followed as explain above). The samples were checked on 12 % SDS-PAGE and Coomassie staining.

Concentration of the proteins were calculated by measuring absorbance of the proteins at 280 nm, based on the predicted extinction coefficient calculated from the amino acid compositions of the proteins using the EXPASY Prot Param server available online (Table 2)

TDH Variants	Extinction coefficient (1 mg/ml)	Isoelectric point (PI)	Molecular weight
WT TDH (With His tag)	1.177	5.57	20852.1
WT TDH (Without His tags)	1.307	4.78	18773.8
Y87A TDH (With His tag)	1.11	5.57	20760.8
Y87A TDH (Without His tags)	1.234	4.78	18681.7
Y87F TDH (With His tag)	1.106	5.57	20836.1
Y87F TDH (Without His tags)	1.229	4.78	18757.8

Table 2: Extinction coefficient, Isoelectric point and Molecular weight of the TDH variants.

6. Functional studies of the recombinant TDH variants:

To check the functional activity of WT TDH, Y87A TDH and Y87F TDH, hemolytic activity of the TDH variants were done against human erythrocytes. Human blood was diluted in PBS (20 mM sodium phosphate buffer and 150 mM NaCl) (pH 7.4) and checked the OD at 650 nm was kept less than 0.9. Then, it was incubated with WT TDH and its variants (concentration of the proteins used was 1.25 μM). The activity of the toxins was determined by decrease in turbidity of the blood. We checked OD at 650 nm for every 5 minutes of incubation, for a total of 1 hour.

7. Far-UV CD and Intrinsic Tryptophan Fluorescence:

In order to determine if WT TDH and its variants have their secondary and tertiary structural integrity, we did far-UV CD and intrinsic tryptophan fluorescence experiments, respectively. Far-UV CD spectra were recorded from 190 nm to 260 nm. Intrinsic tryptophan fluorescence spectra were monitored using excitation wavelength of 290 nm (Slit width for excitation is 2.5 mm). Emission wavelengths were set at 310-450 nm (Slit width for emission is 5 mm). For these experiments, protein concentration used was 2 μM in 5 mM Tris HCl buffer (pH- 8).

8. Pull Down Assay:

8.1. Liposome Preparation:

10 mg Phophatidylcholine, PC (SIGMA) was dissolved in 1 ml chloroform in a round bottom flask and put in a dessicator for 4 to 5 hours. 5 ml of PBS (20 mM sodium phosphate buffer and 150 mM NaCl) (pH 7.4) was added and kept at 37 °C for 3 hours with shaking at 200 rpm.

8.2 Liposome Pull Down Assay:

To check the binding of Y87F TDH and WT TDH with PC liposomes, liposome pull down assay was done. (Y87A TDH was ignored for the experiment)

The procedure is, as follows:

1) Freshly prepared PC liposomes were ultra centrifuged at 80,000 rpm for 40 minutes.
2) Supernatant was discarded and 50 µl of 20 µM protein was added to the pellet.
3) It was incubated at 37 °C for 30 minutes and ultra centrifuged at 80,000 rpm for 40 minutes.
4) Supernatant was collected (proteins which did not bind with the PC liposome would be in the supernatant).
5) Pellet was washed with 50 µl PBS (20 mM sodium phosphate buffer and 150 mM NaCl) (pH- 7.4).
6) Again, it was ultracentrifuged at 80,000 rpm for 40 minutes.
7) Supernatant was discarded and pellet was resuspended at 50 µl PBS (20 mM sodium phosphate buffer and 150 mM NaCl).
8) Equal volumes of supernatant (collected in step (4)) and resuspended pellet (in step 7) were checked for the presence of protein by SDS-PAGE followed by western blotting using anti-TDH.

Images of the blots were taken from ImageQuant LAS4010 (GE healthcare life science)

8.3. RBCs Pull Down Assay:

We further checked the binding of Y87F TDH and WT TDH with human RBCs (Y87A TDH was ignored for the experiment).

The procedure is, as follows:

1) Blood sample in PBS (20 mM sodium phosphate and 150 mM NaCl) (pH 7.4) was washed (centrifuged at 2000 rpm for 25 minutes) several times in PBS till the supernatant became clear.
2) OD (at 600 nm) of the blood in PBS (20 mM sodium phosphate and 150 mM NaCl) (pH 7.4) was kept less than 0.9
3) 500 µl blood was mixed with 500 µl of 2 µM proteins (Y87TDH and WT TDH) and incubated for 1 hour at 37 °C.
4) It was then, ultracentrifuged at 60,000 rpm for 30 minutes.
5) Supernatant was collected and the pellet was washed with 500 µl PBS (20 mM sodium phosphate and 150 mM NaCl) (pH 7.4) by ultracentrifugation at 60,000 rpm for 30 minutes.
6) Supernatant was discarded and the pellet was resuspended in 50 µl PBS (20 mM sodium phosphate and 150 mM NaCl) (pH 7.4).
7) Equal amounts of supernatant (collected in step 5) and resuspended pellet (in step 6) were checked for the presence of proteins by SDS-PAGE followed by western blotting using anti-TDH.

Images of the blots were taken from ImageQuant LAS4010 (GE healthcare life science).

RESULTS

1) Superimposition of TDH and StnII Structures:

PDB ID of TDH (PDB ID: 3A57) and StnII (PDB ID: 1GWY) were retrieved. The two structures were superimposed based on SSM (secondary structure matching) (fig.5). Tyr87 of TDH overlaps with Tyr111 of StnII. As, Tyr111 of StnII is important in binding to POC, Tyr87 of TDH might be involving in binding to cell membranes. In pymol view, we further observed that, as Tyr111 of StnII is surface exposed, similarly, Tyr87 of TDH is also surface exposed (fig.6).

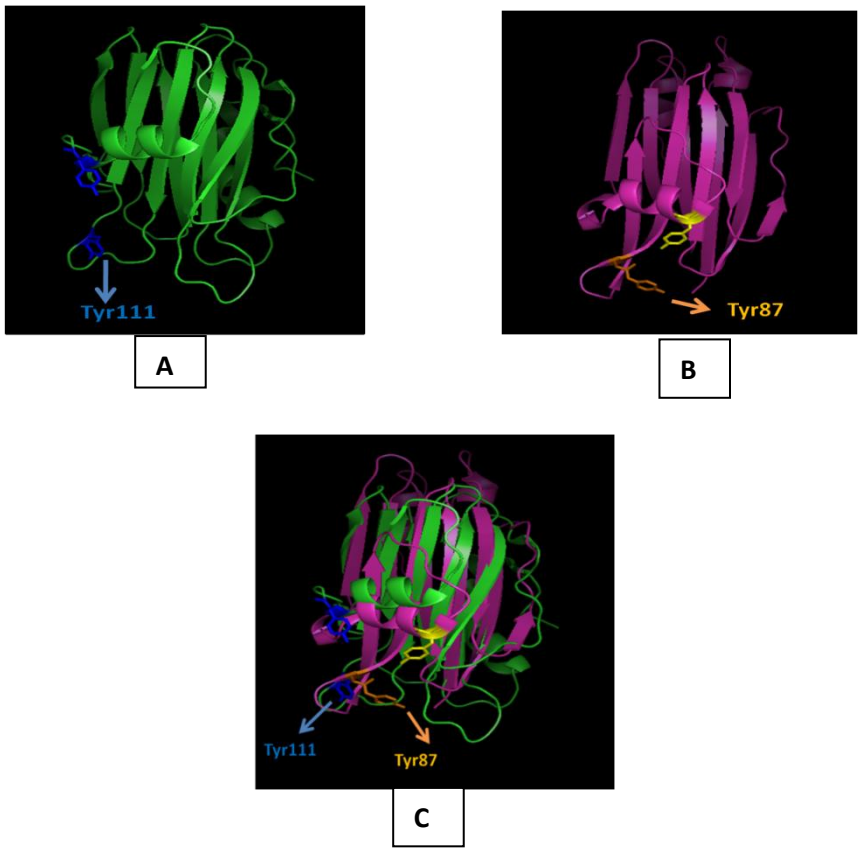


Fig. 4: Pymol view of ribbon representation of A) StnII (PDB ID: 1GWY) showing Tyr111, B) TDH (PDB ID: 3A57) showing Tyr87 and C) Superimposed ribbon representation of StnII and TDH showing overlapping of Tyr111 of StnII and Tyr87 of TDH.

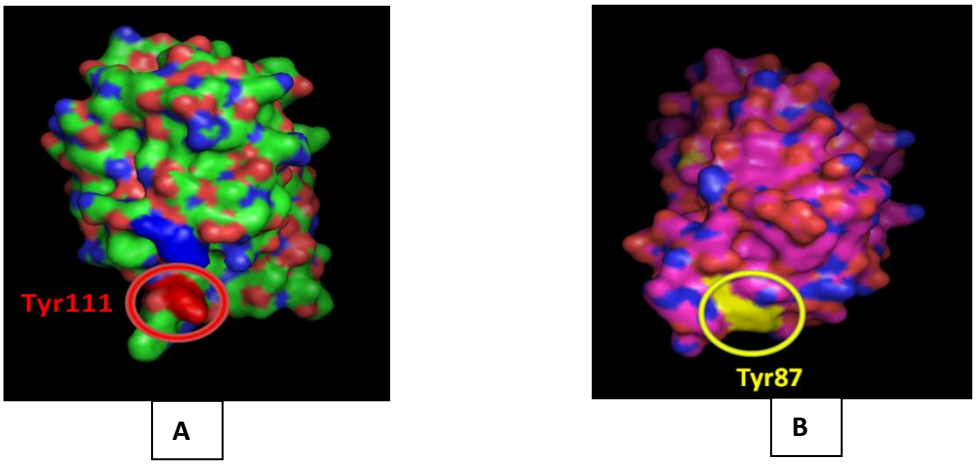


Fig. 5: Surface representation of A) StnII (PDB ID: 1GWY) showing Tyr111 and B) TDH showing Tyr87 (PDB ID: 3A57).

2) <u>Site-directed Mutagenesis for the construction of Y87A *tdh* and T87F *tdh*:</u>

Y87A *tdh* and Y87F *tdh* were constructed using PCR-based method. The figure below shows the amplified PCR products of WT *tdh*, T87A *tdh* and Y87F *tdh* on 1% agarose gel. Both the constructs were checked and verified by DNA sequencing.

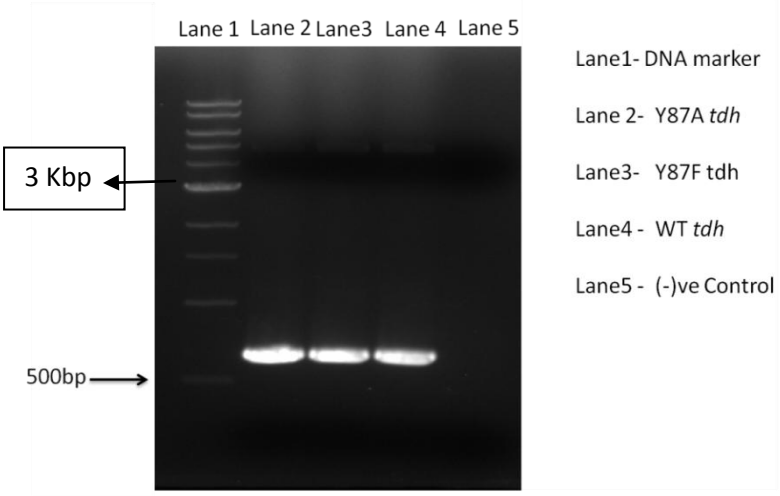


Fig. 6: Agarose gel indicating amplified PCR Products. In lane 5, only primers and mastermix were added (without the template) as negative control.

3) Small Scale Expression Check of WT TDH, Y87A TDH and Y87F TDH:

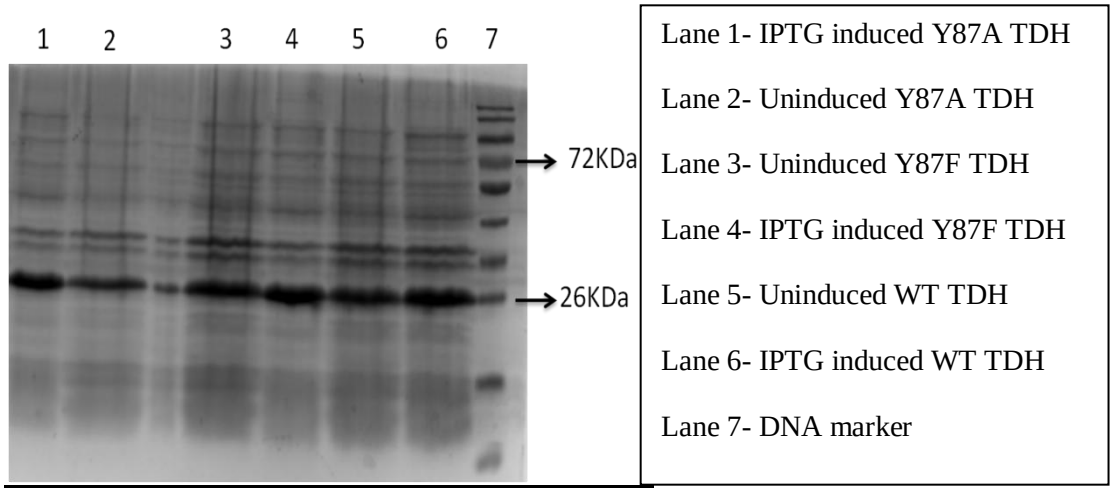


Fig. 7: SDS PAGE/ Coomassie staining showing the expression of Y87A TDH, Y87F TDH and WT TDH.

Expression of the proteins in *E. coli* Origami cells were checked after induction with IPTG. IPTG induction leads to over expression of the proteins.

4) Purification of WT TDH, Y87A TDH and Y87F TDH:

Two rounds of purification were done for the purification of proteins. Ni-NTA affinity chromatography was performed, followed by Q-sepharose anion exchange chromatography. His-tag incorporated with the proteins was cleaved by thrombin treatment.

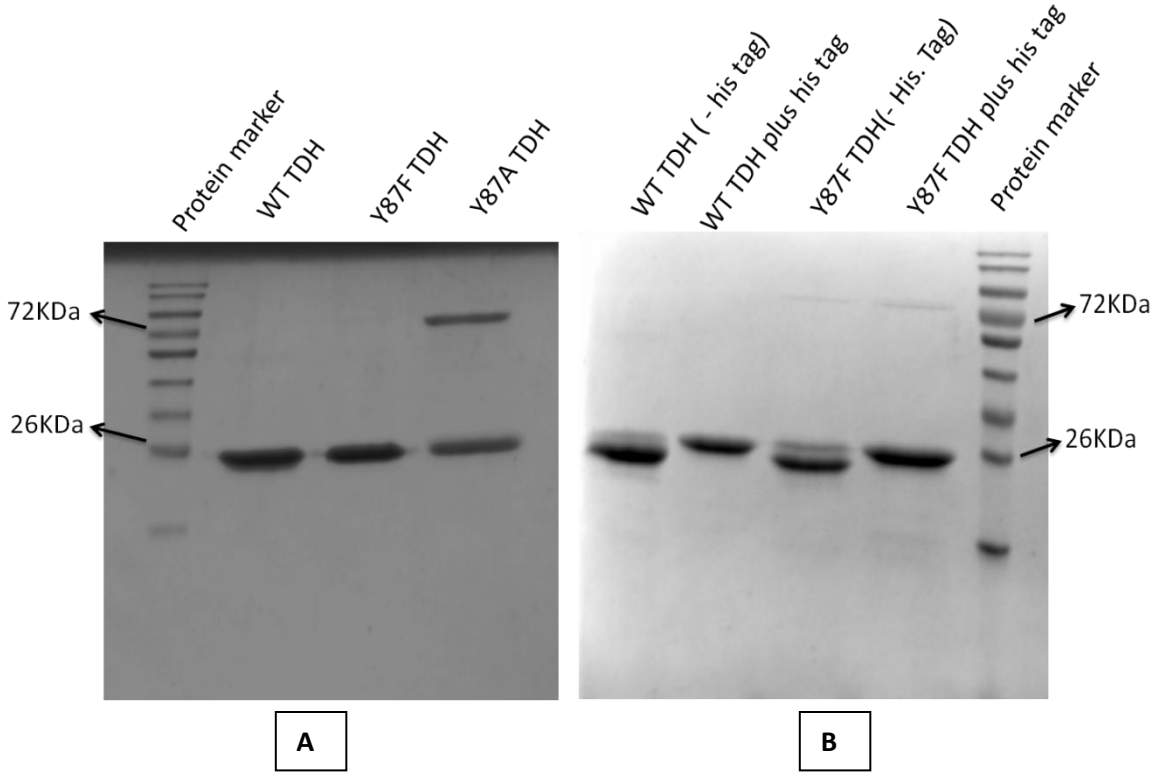


Fig. 8: A) SDS PAGE/ Coomassie staining of purified WT TDH, Y787A TDH and Y87F TDH, B) SDS-PAGE/ Coomassie staining of WT TDH and Y87F TDH after removal of His-Tag.

5) Hemolytic Activity Assay of WT TDH, Y87A TDH and Y87F TDH:

Functional activities of the TDH variants were checked by incubating the toxins with human erythrocytes. WT TDH shows 100 % hemolysis, while there are significant decreased in hemolysis of RBCs when incubated with Y87A TDH and Y87F TDH.

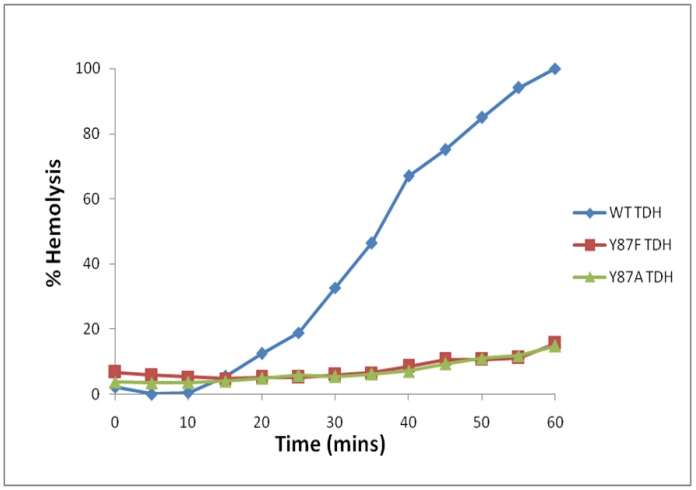
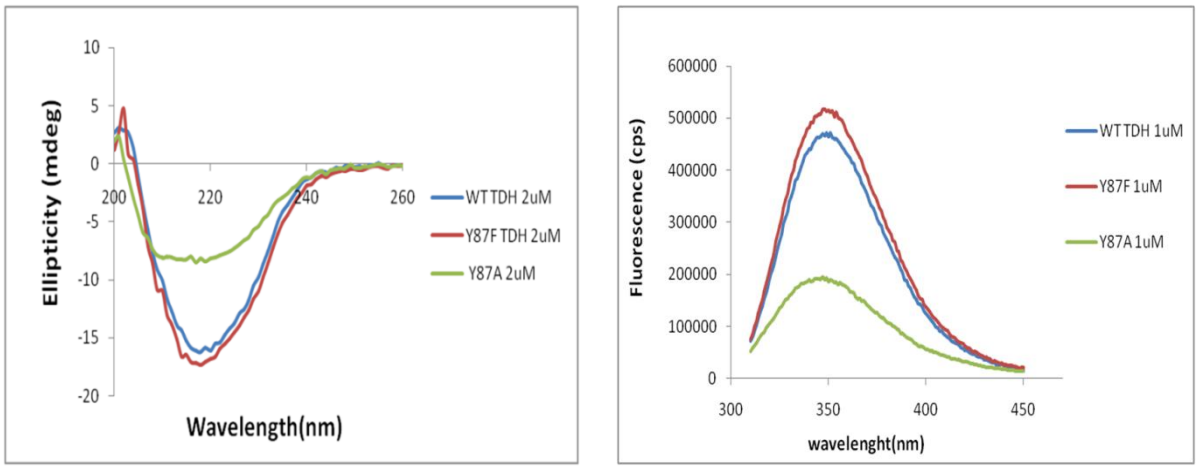


Fig. 9: Hemolytic activity assay of TDH variants. Human erythrocytes were incubated with the toxins and the kinetics of the hemolysis was observed by taking optical density (at 650 nm) at different time points.

6) <u>Far-UV Spectra and Intrinsic Tryptophan Fluorescence Spectra of WT TDH, Y87A TDH and Y87F TDH:</u>



A B

Fig.10: A) Far-UV Spectra (190 nm- 260 nm) of TDH variants, B) Intrinsic tryptophan fluorescence spectra (excitation wavelength at 290 nm, excitation slit width at 2.5 mm, emission slit width at 5 mm) of TDH variants.

In order to check if TDH variants, Y87A TDH and Y87F TDH, maintain secondary structures and to check if both the mutants are properly folded as that of WT TDH, we analyzed far-UV spectra and intrinsic tryptophan fluorescence spectra, respectively. From the far UV spectra, and intrinsic tryptophan fluorescence, it was observed that the structural integrity of Y87F TDH has been maintained but there has been deviation in Y87A TDH as that of WT TDH.

7) <u>Pull Down Assay:</u>

To check the binding of TDH variants in the PC liposome and RBC, Pull Down assay was done (both with PC liposome as well as with RBCs). PC liposome and RBCs were incubated with the TDH variants and ultracentrifuged. If the toxins bind to the membrane, then the bounded proteins would be present in the pellet fraction. From these experiments, it was observed that Y87F TDH binds to both PC liposome and RBCs similarly as that of WT TDH.

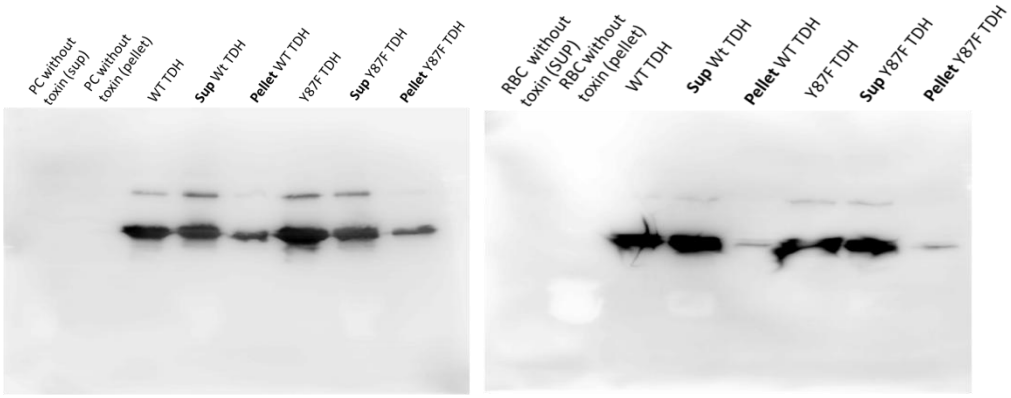


Fig. 11: A) **Pull down assay with PC liposome**. TDH variants were incubated for 30 mins. at 37 °C followed by ultracentrifugation, 80,000 rpm for 40 mins., pellet was washed with PBS and ultracentrifuged, pellet was resuspended in PBS followed by SDS-PAGE/western blotting with anti-TDH. B) **Pull down assay with RBCs**. TDH variants were incubated for 1 hr at 37 °C followed by ultracentrifugation, 60,000 rpm for 30 mins. Pellet was washed with PBS and ultracentrifuged, pellet was resuspended in PBS followed by SDS-PAGE/western with anti-TDH.

CONCLUSION

From the hemolytic activity assay, it is observed that Y87A TDH and Y87F TDH lose their functional activities, while WT TDH shows 100 % hemolysis of RBCs. From far-UV spectra, and intrinsic tryptophan fluorescence spectra, it is observed that Y87F TDH maintained structural integrity, while there is deviation in Y87A TDH as that of WT TDH. Y87F TDH shows similar binding as that of WT TDH with PC liposome and RBCs. From the present results, it could not be concluded that Tyr87 in TDH is important in membrane binding. But, it is certain that Tyr87 in TDH is important in formation of a functional toxin as, both Y87A TDH and Y87F TDH lose their functional activity when checked with human erythrocytes.

References:

1) Gilbert R J C (2002) Pore forming toxin. Cell. Mol. Life Sci. 832-844. (Review)
2) Blake PA, Weaver RE, Hollis DG (1980) Diseases of humans [other than cholera] caused by vibrios. Annu Rev Microbiol. 34: 341- 367.
3) Shinoda S (2011) Sixty years from the discovery of *Vibrio parahaemolyticus* and Some Recollections. Biocontrol Science. 16: 129- 137. (Review)
4) Raghunath Pendru. (2014) Roles of thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) in *Vibrio parahaemolyticu*. Front Microbiol. 805: 1- 4.
5) Park KS, Ono T, Rokuda M, Jang M, Okada K, Iida T, Honda T (2004) Functional Characterization of Two Type III Secretion Systems of *Vibrio parahaemolyticus*. Infect Immun. 72: 6659-6665.

6) Miwatani T, Takeda Y, Sakurai J, Yoshihara A, Taga S (1972) Effect of Heat (Arrhenius Effect) on Crude Hemolysin of *Vibrio parahaemolyticus.* Infect Immun. 6: 1031-1033.
7) Honda T, Ni Y, Miwatani T, Adachi T, Kim J (1992) The thermostable direct hemolysin of *Vibrio parahaemolyticus* is a pore-forming toxin. Can J Microbiol. 38: 1175-1180.
8) Hamada D, Higurashi T, Mayanagi K, Miyata T, Fukui T, Iida T, Honda T, Yanagihara I (2007) Tetrameric structure of thermostable direct hemolysin from Vibrio parahaemolyticus Revealed by Ultracentrifugation, Small-angle X-ray Scattering and Electron Microscopy. J. Mol. Biol. 365: 187-195.
9) Shirai H, Ito H, Hirayama T, Nakamoto Y, Nakabayashi N, Kumagai K, Takeda Y, Nishibuchi M (1990) Molecular epidemiologic evidence for association of thermostable direct hemolysin (TDH) and TDH-ralated hemolysin of Vibrio parahaemolyticus with gastroenteritis. Infect Immun. 58: 3568-3573.
10) Kishishita M, Matsuoka N, Kumagai K, Yamasaki S, Takeda Y, Nishibuchi M (1992) Sequence variation in the thermostable direct hemolysin-related hemolysin (trh) gene of Vibrio parahaemolyticus. Appl. Environ. Microbiol. 58: 2449-2457.
11) Tsunasawa S, Sugaihara A, Masaki T, Sakiyama F, Takeda Y, Miwatani T, Narita K (1987) Amino acid sequence of Thermostable Direct Hemolysin Produced by *Vibrio parahaemolyticus.* J Biol Chem 101: 111-121.
12) Yanagihara I, Nakahira K, Yamane T, Kaieda S, Mayanahi K, Hamada D, Fukui T, Ohnishi K, Kajiyama S, Shimizu T, Sato M, Ikegami T, Ikeguchi M, Honda T, Hashimoto H (2010) Structure and Functional Characterization of Vibrio parahaemolyticus Thermostable Direct Hemolysin. J Biol Chem. 285: 16267-16274.
13) Shekar M, Karunasagar I (2013) Structural analysis of *Vibrio parahaemolyticus* thermostable direct hemolysin and insight into its carbohydrate recognition, Internation Journal of computational Bioinformatics and In Silicon Modeling 2: 269-274.

14) Mancheno JM, Martín-Benito J, Martínez-Ripoll M, Gavilanes JG, Hermoso JA (2003) Crystal and electron microscopy structures of sticholysin II actinoporin reveal insights into the mechanism of membrane pore formation. Structure 11: 1319– 1328.

15) Ferdinand COL, Tara MR, Aroian RV, Ratner AJ (2013) Role of Pore-Forming Toxins in Bacterial Infectious Diseases. Microbiol Mol Bio Rev. 77: 173- 207. (Review)

16) Lucia GO, Jorge AC, Sara GL, Marta B, Alvaro M P, Jose GG (2011) The behavior of Sea anemone actinoporins at the water-membrane interface. Biochimica et Biophysica Acta 1808: 2275- 2288. (Review)

.