The pH-induced structural changes in *Helicobacter pylori* TlyA

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

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CERTIFICATE

This is to certify that the dissertation titled "**The pH-induced structural changes in *Helicobacter pylori* TlyA**" submitted by Ms. Amritha Sreekumar (Reg. No. MS11037) for the partial fulfilment 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.

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(Supervisor)

Dated: April 22, 2016

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.

Amritha Sreekumar
(Candidate)
Dated: April 22, 2016

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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I would like to deeply thank those who have helped me to complete this project with happiness and satisfaction.

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ABSTRACT

Helicobacter pylori TlyA is a novel pore-forming toxin which is amyloidogenic in nature. Mode of action and virulence mechanism of this toxin remain obscure. However, cytotoxicity of its amyloid fibrils over a range of temperature has given new insights toward this. Our understanding of protein behavior in other physiologically relevant environment would take us rather close to its virulence mechanism. We know that *Helicobacter pylori* (HP) survive in extremely low pH for being a gastric pathogen. Furthermore, it has been shown that HP inflammation causes acid production in stomach. Hence we studied the structural changes in *H. Pylori* TlyA brought about by low pH environment.

In this study, we observed that even at the pH of 3 the protein retains its secondary structure intact and shows only sparse amyloid fibril formation. The protein shows tremendous ANS binding upon low pH incubation suggesting a prominent conformational change, exposing its hydrophobic patches on the surface. The protein in reinstated neutral pH showed significantly low ANS binding presumably due to the refolding of the protein. The functionality of the native and refolded protein has been studied via hemolytic assay. In this assay we show that the refolded protein achieves a higher activity than the native protein. Finally, our data suggest pH as one of the major physico-chemical constraint for the virulence activity of the protein. Altogether we report that *H. Pylori* TlyA has a propensity to reversibly unfold over a range of acidic pH while retaining the structural integrity and functionality to a great extent.

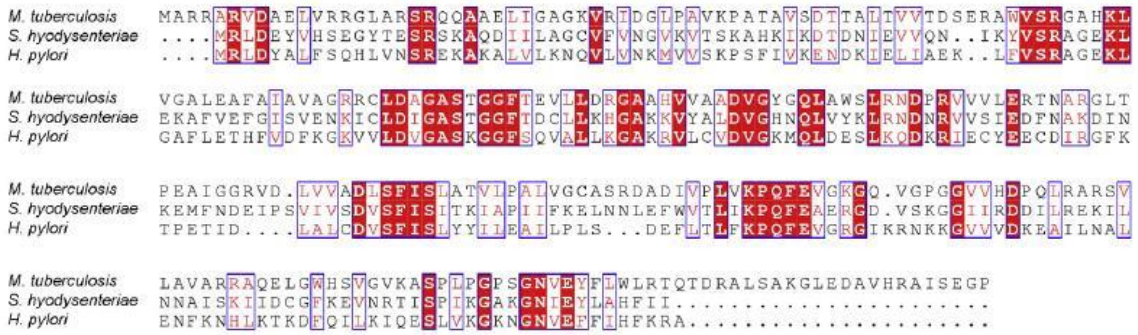
CHAPTER 1

Introduction

1.1 Background

Helicobacter pylori is a Gram negative, human specific gastric pathogen which is one of the most evolutionarily successful bacteria that infects half of the human population. It survives in the harsh acidic environment of stomach and colonises in mucosal lining of the stomach. More than 80% of the infections are asymptomatic. This insinuates the adaptability and survival fitness of the bacteria as a keystone microbe in the stomach ecology. Nonetheless, pathogenecity of the bacteria has been proven beyond doubt and it won its discoverers the Nobel in 2005. It is the only microbe ranked as a carcinogen. The survival of these bacteria in an environment that is critically severe is aided by its arsenal of virulence factors and its ability to manipulate host's immune system. It has been shown that in an asymptomatic, HP positive person, the inflammatory response due to other pathogen attack is augmented. The major virulence factors of these bacteria have been identified as CagA and VacA. However the *H. pylori* determinants responsible for inducing inflammation during gastritis are still enigmatic to us. Hence characterization of additional virulence factor is necessary. Analysis of HP genome has led to the identification of two putative hemolysins – HP 1086 and HP 1490. The former has approximately 35% sequence similarity to the TlyA proteins of *M. tuberculosis* and *S. hyodysenteriae*, hence a good candidate as another virulence factor (Fig. 1A). HP 1086, now popularly known as *Helicobacter pylori* TlyA (*H. pylori* TlyA) is a potent pore-forming hemolysin. The structural and functional characterization of *H. Pylori* TlyA in its purified has revealed that it has the ability to lyse human erythrocytes presumably via pore-formation. It exhibits potent cytotoxicity against human gastric

adenocarcinoma (AGS) cells. Moreover, it shows amyloidogenic property over a wide range of temperature, including, physiologically relevant temperature 37 ^{o}C.



Lata *et al.*, 2014

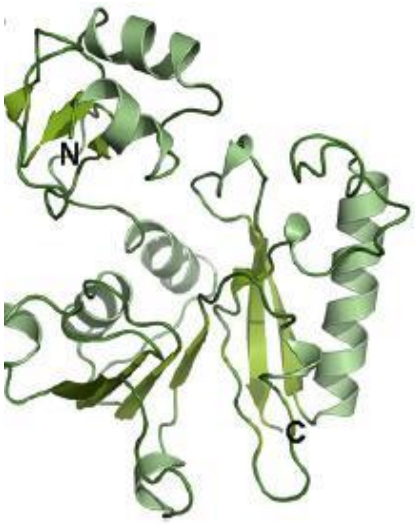
Figure 1 (A): Multiple sequence alignment of *H. Pylori* TlyA with TlyA-like proteins from *M. tuberculosis* and *S. hyodysenteriae*. *H. pylori* TlyA amino acid sequence shows 33% and 36% sequence identity with the TlyA –like protein from *M. tuberculosis* and *S. hyodysenteria*, respectively.

However, the known functionalities of *H. Pylori* TlyA are not adequate enough to make plausible conclusion toward the HP pathogenesis process. A comprehensive understanding of protein behavior at physiologically relevant condition can divulge more physico-chemical constraints associated with the mode of action of *H. Pylori* TlyA.

1.2 Motivation and question

We know, during initial stages of infection, survival of *Helicobacter pylori* in stomach lumen is ensured by the urease-dependent ammonia production that locally raises the pH at the mucosal layer, where the colonisation occurs. Nevertheless, it survives the extreme pH of 1-2 at stomach lumen for few minutes before escaping the acidic pH. The bacteria undergo an intense pH transition during infection and colonization. This transition may play a role in the virulence behaviour of the bacteria. Moreover, it has been reported that the HP infection increases acid production in stomach. In this thesis, we chose to study the behaviour of *H. Pylori*

TlyA in this challenging environment of low pH. We intended to study the secondary and tertiary structural organisation of HP TlyA at low pH and at the transition from acidic to neutral pH. Furthermore, we compared and analysed the functionality of the protein that has undergone structural alteration at low pH against the native protein (Fig. 1B).



Lata *et al.*, 2014

Figure 1 (B): Homology-based protein structural model of *H. pylori* TlyA depicting its N- and C-terminal.

CHAPTER 2

MATERIALS AND METHODS

2.1 Expression and purification of *H. Pylori* TlyA

The recombinant form of *H. pylori* TlyA was over expressed in *Escherichia coli* Origami B cells (Invitrogen). The protein was purified using 6xHis-tag dependent Ni-NTA chromatography followed by ion exchange chromatography. *E. coli* Origmi B cells harboring the recombinant plasmids were grown in four litres of LB broth containing ampicillin (50 μg/ml) at 37^{o}C under constant shaking at 180 rpm. The cells were induced at A_{600} of ~1.0 with 0.5 μM isopropyl-β-d-thiogalactopyranoside (IPTG) for 5 h at 20^{o}C. Cells were harvested by centrifuging at 4000 rpm and resuspended in PBS buffer containing 20 mM sodium phosphate, 150 mM NaCl. For purifying protein, cells were lysed using ultrasonic disruption method using sonicator. Soluble fraction of the bacterial cell lysate was adjusted with 20 mM imidazole, so as to equilibrate it with Ni-NTA agarose slurry. This ensures effective binding of protein to the beads. The elution of the His-tagged TlyA protein has been done with 200 mM imidazole in PBS. Eluted protein was diluted 7-fold with a 50 mM sodium phosphate buffer (pH 7) and passed through SP Sepharose cation-exchange resin. The bound TlyA protein was eluted with 400 mM NaCl in 50 mM sodium phosphate buffer (pH 7). Homogeneity of the protein was examined by SDS-PAGE and Coomassie staining (Fig. 2).

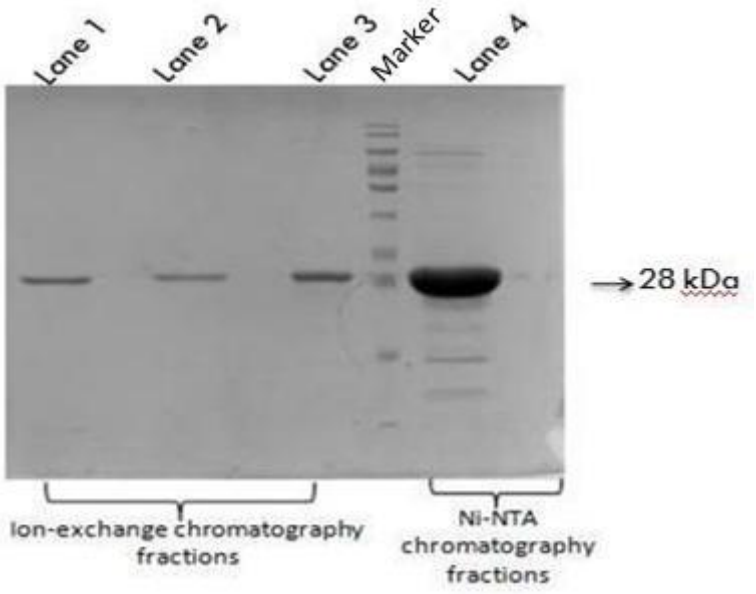


Figure 2: SDS-PAGE/Coomassie staining of purified *H. pylori* TlyA by Ion-exchange and Ni-NTA chromatography. Lanes 1, 2 and 3 shows elusion profile of ion-exchange chromatography. The lane 4 shows elusion profile of Ni-NTA chromatography.

The protein concentration was estimated by absorbance at 280 nm based on the theoretical extinction coefficient calculated from the primary amino acid sequence of the recombinant TlyA (0.21 for 1mg/ml His-tagged TlyA protein).

2.2 Far-UV Circular Dichroism (CD) Spectroscopy

Far-UV CD spectra for the samples were collected on a Chirascan spectropolarimeter. The samples were prepared by incubating *H. pylori* TlyA (2μM) in sodium phosphate buffer at 5 mM citrate buffer in corresponding pH ranging from 3 to 7 for 1 hour. The final spectra were obtained after averaging the collected spectra and subsequent buffer subtraction. The samples were brought to required pH via two methods. In first method, 2 ml reaction mixture was adjusted to the desired pH by adding the appropriate volume of 1 M citrate buffer of corresponding pH. In the second method, pH of the reaction volume was adjusted by buffer-exchange via dialysis method.

2.3 Fluorescence Measurements

All the fluorescence measurements were recorded on Fluoromax-4 (Horiba Scientific) spectrofluorimeter equipped with peltier-based temperature controller. All the data are normalised with respect to the fluorescence intensity maxima value in each set of data.

2.3.1 ANS (1-anilinonaphthalene-8-sulfonicacid) Fluorescence

All the ANS fluorescence measurements were recorded in 50 mM citrate buffer at pH ranging from 3 to 7, using a final ANS concentration of 10 μM in the solution. Steady state fluorescence emission was recorded upon excitation at 350 nm, at an excitation and emission slit widths of 2.5 nm. The spectra were recorded over wavelength range of 400-600 nm. ANS fluorescence spectra were collected at 20^{o}C after a low pH exposure of 1 h. ANS fluorescence for checking refolding of the protein was collected after bringing back the low pH systems to a neutral pH of 7. All the ANS fluorescence spectra were corrected by subtracting those of the protein samples in the absence of ANS. ANS fluorescence in the absence of TlyA served as blank.

2.3.2 ThT (Thioflavin T) Fluorescence

ThT fluorescence of the samples was collected in 50 mM sodium phosphate buffer, at a pH of 7. *H. pylori* TlyA (10 μM) incubated at a low pH was brought back to pH 7 by buffer exchange via dialysis in 50 mM sodium phosphate buffer (pH 7). The spectra were recorded over a wavelength range of 465-550 nm at an excitation wavelength of 450 nm with an excitation and emission slit widths of 2.5 and 5 nm, respectively.

ThT fluorescence spectra were collected at 20^{o}C after a low pH exposure of 3 h. All the ThT fluorescence spectra were corrected by subtracting those of the protein samples in the absence of ThT. ThT fluorescence in the absence of TlyA served as blank.

2.3.3 Nile Red Fluorescence

Nile Red fluorescence was taken for *H. pylori* TlyA (10 μM) was incubated at 50 mM citrate buffer of pH 3 and 6. The final concentration of Nile Red in solution was 2.5 μM. The spectra were collected over a range of 575-750 nm at an excitation wavelength of 550 nm with excitation and emission slit widths of 1 and 2 nm, respectively. Nile Red fluorescence spectra were collected at 20^{o}C after a low pH exposure of 3 h. All the Nile Red fluorescence spectra were corrected by subtracting those of the protein samples in the absence of Nile Red. Nile Red fluorescence in the absence of TlyA served as blank.

2.4 Assay of the lytic activity of *H. Pylori* TlyA against human erythrocytes

The native and refolded protein was diluted in sodium phosphate buffer (50 mM, pH 7), mixed with human erythrocytes suspended in PBS for 18 h at 25^{o}C. Human erythrocytes concentration in the reaction mixtures were adjusted such that complete lysis of the cells corresponded to the Absorbance at 415 nm (A_{415}) of 0.9. After incubation of toxin with human erythrocytes, cells were centrifuged and the absorbance of supernatant was measured at 415 nm. The 100% lysed erythrocytes upon treatment with 100 nM *Vibrio cholera* cytolysin served as control.

CHAPTER 3

RESULTS

3.1 Incubation of *H. pylori* TlyA at low pH does not alter the secondary-structural organisation of the toxin

Far-UV CD spectroscopy has been used to study the secondary structure of the protein. For this analysis, we incubated purified *H. Pylori* TlyA in five different pH conditions, at an acidic pH range. The protein samples incubated at different pH conditions produced spectra that overlapped to a good extent (Fig. 3A).

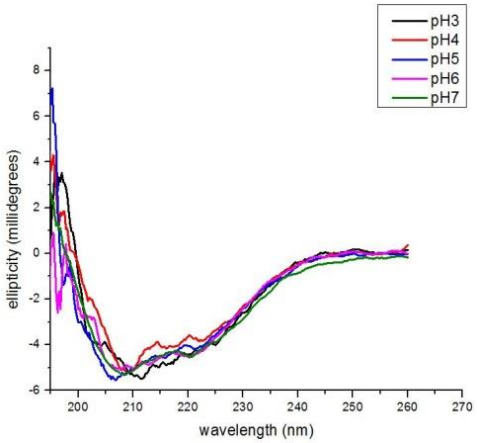


Figure 3 (A): Far-UV CD spectrum of HP TlyA under acidic pH condition. Spectra generated at different pH values indicate that there is no significant change in the overall secondary structure of the protein.

The spectra showed no prominent shifts in the peak-maxima. This data suggest that the protein retains an intact secondary structural organisation at acidic pH environment. All the spectra showed a broad spectrum of negative ellipitcity between the wavelengths of 208 and 222 nm, with the negative ellipticity value peaking at

around 208 nm. As earlier stated, pH incubation of the protein has been done in two methods. Both the methodologies produced same results.

3.2 The low pH environment elicits reversible unfolding of *H. pylori* TlyA

ANS fluorescence assay has been used to study the conformational changes at a global tertiary structural level. In the initial stage, the protein samples exposed to low pH environment were used for the fluorescence measurements. ANS fluorescence emission maxima were observed at ~470 nm. The data suggested a tremendous conformational change, presumably unfolding of protein, exposing the hydrophobic patches on its surface at the lowest pH i.e. pH 3 (Fig. 3B). The ANS fluorescence emission dropped significantly when the pH of the system gradually increased.

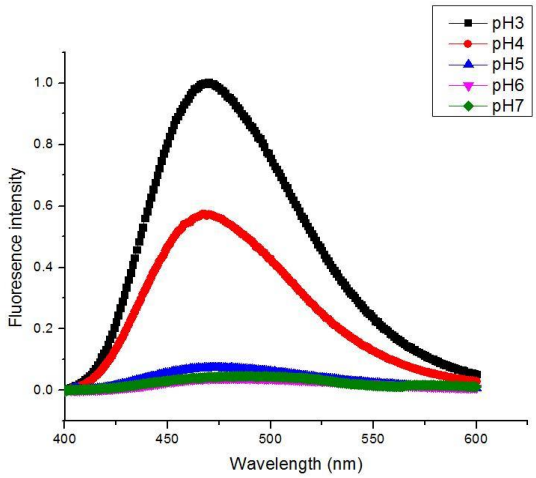


Figure 3 (B): Changes analysed in the global structure of the protein by ANS fluorescence assay. At low pH condition *H. pylori* TlyA unfolds thereby exposing its hydrophobic patches. The protein tends to remain stable at pH 7, 6 and 5 but unfolds as the pH drops below 4. Maximun conformational change occurs at lowest pH.

Although the protein shows an extensive unfolding, the stable secondary structure of the same was intriguing. Hence, we checked the reversibility of the conformational change after reinstating the system with the favourable neutral pH. We used a buffer-exchange method via dialysis to accomplish this. The ANS fluorescence measurements of the samples were collected. The spectra obtained showed significant

drop in the fluorescence emission upon buffer-exchange. (Fig. 3C). The samples that are brought back to pH 7 from the lower pH 3 and 4 showed a conspicuous drop in the fluorescence emission, suggesting an efficient refolding of the protein. Clearly, a 4 to 7 transition shows efficient refolding compared to 3 to 7 transition.

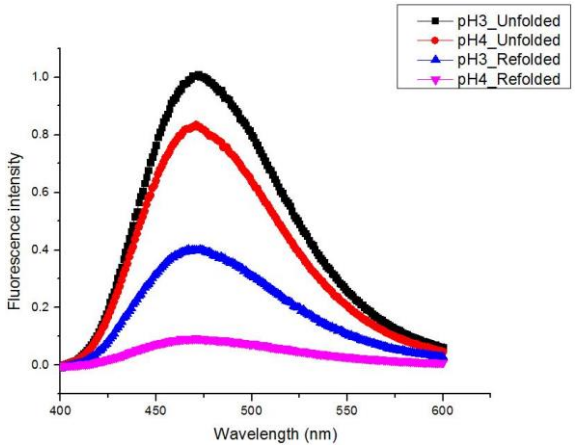


Figure 3 (C): Changes analysed in the global structure of the refolded protein by ANS fluorescence assay.At pH 3 and 4, HP TlyA unfolds and hence give high ANS fluorescence at same pH.When protein incubated with the buffer of pH 3 and 4, brought back to the neutral environment i.e. pH 7, it showed less ANS fluorescence indicating that protein has refolded in pH 7 buffer.

3.3 Nile Red assay detects amyloid fibril formation upon low pH incubation

Nile Red is a pH-insensitive dye which gives fluorescence upon binding specifically to the amyloid-like fibrils. This assay has been conducted as a preliminary test for the detection of amyloid fibril formation. Over the acidic pH range, this assay has been done for the lowest and highest pH, i.e., 3 and 6.

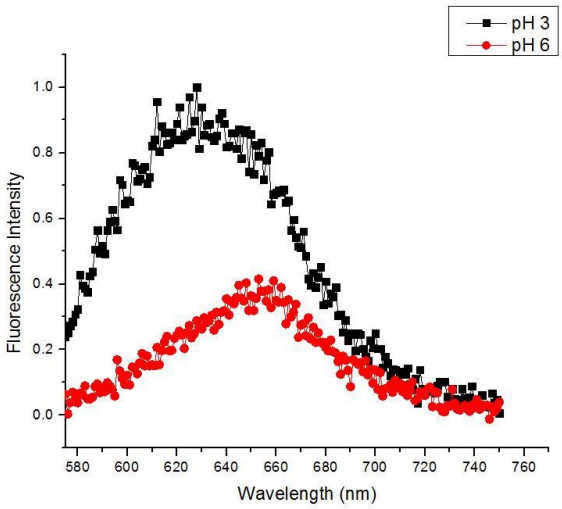


Figure 3 (D): Determination of amyloid like fibrils formation by Nile Red assay. At pH 3, *H. pylori* TlyA shows high fluorescence intensity compared to pH 6, indicating clear difference in the amyloid formation propensity of the protein at both the pH conditions.

The samples for fluorescence measurements were prepared by incubating *H. pylori* TlyA at a pH of 3 and 6 for 3 h. The low pH induced a higher Nile Red fluorescence emission. The protein samples at both the pH gave a substantial difference in the fluorescence emission (Fig. 3D). The pH 3 incubation has triggered more amyloid-fibril formation rather than pH 6 incubation. Hereby we know that pH plays a role in the amyloidogenecity of the protein.

3.4 The low pH incubation of H. pylori TlyA triggers stable amyloid-like fibril formation

ThT fluorescence gives more reliable data for the identification of amyloid-formation. However, ThT is a pH-sensitive dye which is not active at acidic pH range. Hence, we checked the extent of amyloid formation in the protein samples after readjusting the system to a neutral pH (Fig. 3E). For this experiment, samples were incubated at low pH conditions for 3 hours.

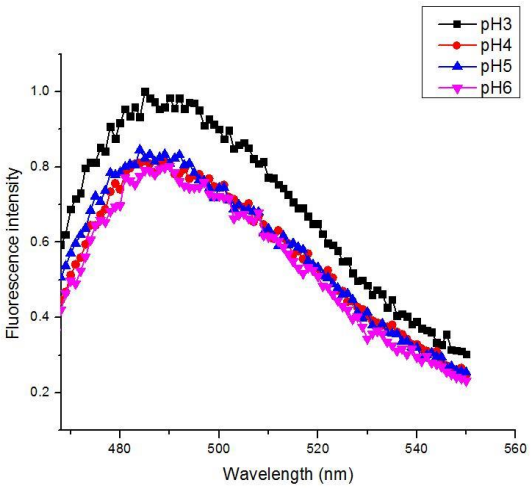


Figure 3 (E): Determination of amyloid like fibrils formation by ThT fluorescence assay. At pH 3, TlyA shows high fluorescence intensity compared to pH 6, indicating maximum amyloid formation at pH 3. Spectra collected over a pH from 4 to 6 have overlapped. This suggests that amyloid-like fibril formation over this pH range remained constant.

The data from ThT fluorescence is in agreement with the Nile Red fluorescence data. *H. pylori* TlyA shows highest propensity for amyloid formation at the lowest pH condition. Under rest of the higher pH conditions, the ThT fluorescence emission remained same.

3.5 Refolding enhances haemolytic activity of *H. pylori* TlyA against human erythrocytes

The structural changes in proteins are profound in the light of functional implications associated with it. Therefore, we compared the functionality of refolded protein against the native protein. We studied the ability of the proteins to lyse human erythrocytes. In our experiment, we found that, refolded protein is functionally active. Up until a concentration of 5 μM, the refolded protein shows 15% lysis, lesser to the native protein by only 10% (Fig. 3F). However, at a concentration of 10 μM, the activity of the refolded protein increases considerably, suggesting an enhanced functionality via refolding.

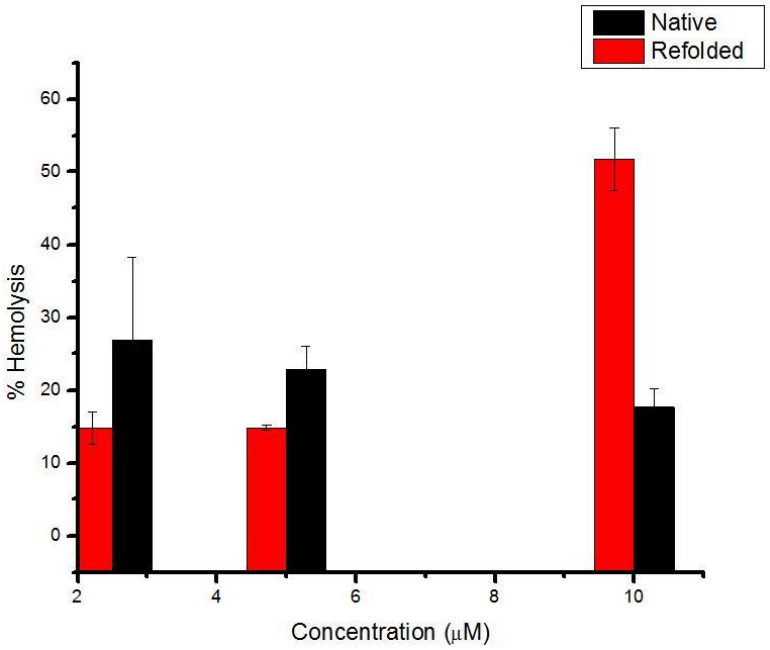


Figure 3 (F): Concentration-dependent hemolytic activity of the native and refolded TlyA against human erythrocytes in hemolytic assay. At a concentration of 10 μM, the refolded TlyA shows enhanced hemolytic activity against human erythrocytes compared to native protein.

CHAPTER 4

DISCUSSION

The aim of this study was to establish low pH environment as a physico-chemical constraint for the mode of action *H. pylori* TlyA. We analysed the secondary and tertiary structural organisation of the protein. We used Far-UV CD spectroscopy for the former and ANS fluorescence assay for the later. We also analysed amyloidogenesity of the protein under low pH exposure using Nile Red fluorescence assay and ThT fluorescence assay.

At low pH environment for a majority of bio-chemical processes are altered. In our experiments, we speculated that protein samples might undergo extensive conformational changes upon low pH incubations. However, the CD spectroscopy data we obtained was intriguing for its unaltered peak maxima for all the protein samples. The CD spectra of all the samples overlapped to a good extend (Fig. 3A). This data shows a structurally intact secondary conformation. This result dismisses the probability of extensive amyloid-like fibril formation in low pH condition. In case of amyloid formation, we see a prominent shift in the negative ellipticity minima toward the wavelength region of 219-221 nm.

Further, in the ANS fluorescence assay the global tertiary structure of protein was characterised. Upon decreasing the pH, a steady increase in the ANS fluorescence emission has been observed. This suggests pH dependent unfolding of the protein, exposing the hydrophobic patches on its surface. It is interesting to note that, at pH as high as 5 and 6, TlyA shows insignificant ANS fluorescence emission. An abrupt increase in the fluorescence emission is observed at pH 3 and 4. We can speculate the increased fluorescence emission is owing to a prominent conformational change which alters the global tertiary organisation of the protein. The results tempt us to assume that the protein has undergone a conformational change to attain a molten

globule stage. In this stage, TlyA has a conserved native-like secondary structure but the interior of the protein lacks a tight packing. A weakly packed protein interior would eventually lead to the exposure of hydrophobic patches on the surface. This stage is far different from the native as well as the denatured state of the protein. It has been studied that, at a favourable condition, molten-globule conformation re-organise to a native- like structure. The steady drop in the ANS fluorescence emission upon readjusting the neutral pH, presumably owing to the refolding of the protein further strengthens the argument of molten globule formation.

Amyloid fibril formation has been identified as a hallmark character of the HP TlyA. A sparse amyloid formation has been detected in the low pH environment could be interpreted as the confirmation of this fact. Nile Red assay is not regarded as an efficient tool for the identification of amyloid-fibrils owing to its inconsistency in the peak shifts at low pH. However, the data we obtained clearly suggests that there is a clear demarcation in the amyloid formation in TlyA at different pH condition. This is consistent with the data of ThT fluorescence assay. A detailed study will be necessary to elucidate the cytotoxic potential of the scarce amyloid fibril that has formed upon pH incubation. The scarce amyloid-formation in a challenging environment like extreme low pH, further strengthens the possibility of molten globule-formation of *H. pylori TlyA* at low pH.

The structural changes brought about by a set of condition could have functional implications. Here we analysed the ability of the native and refolded protein to lyse human erythrocytes. In this experiment we observed, the refolded protein not only remain active but also shows an enhanced activity towards the human erythrocytes at a concentration of 10 μM. The hemolytic activity of *H. pylori* TlyA has been attributed to the pore-formation on the membrane. The enhanced activity of the refolded protein let us speculate that the structural rearrangements during the refolding facilitate effective membrane pore-formation. A detailed study is necessary to substantiate and elucidate the enhanced activity of the refolded protein toward the haemolytic activity.

CHAPTER 5

CONCLUSION

In this study, we performed structural and functional characterisation of protein at a physiologically relevant environment that is the low pH. Usually an acidic pH range is a hostile environment for most the organisms. However for *Helicobacter pylori*, acidic environment is part of its niche for being a human gastric pathogen. TlyA has been characterised as a potent virulence factor of *Helicobacter pylori*.

In this study, we have demonstrated the pH dependence of *H. Pylori* TlyA for its structural and functional alteration. We have shown that even at the challenging low pH, secondary structure of the protein remains intact. However a sparse amyloid formation has been found in associated with low pH incubation. TlyA shows a strong reversible unfolding, opening the hydrophobic patches, upon the low pH incubation. The reinstated neutral pH led to the efficient refolding of the protein back to the native form. On the basis of the results of structural organisation upon subjecting the protein to a low pH and the pH- transition, it is possible to speculate that *H. pylori* TlyA undergo a molten-globule structural alteration under this condition. The refolded protein appears to have an enhanced functionality, that is, the ability to lyse human erythrocytes. Perhaps the re-organisation of protein potentially contributes to the virulence mechanism of the *Helicobacter pylori*. This study establishes low pH as a significant physico-chemical constraint for the mode of action *H. Pylori* TlyA

CHAPTER 6

FUTURE PROSPECTS

Although the preliminary data that we have shown proposed an enhanced hemolytic activity of the refolded protein, an elaborate functional characterization of refolded protein is required to make a conclusive remark on the activity of the protein. LDH release assay for native and refolded protein can further prove the functional implications of the conformational change brought about by the pH-transition.

In this project, we explored the protein behavior in an acidic pH range. However, for a comprehensive understanding of the role of pH in the folding-refolding of TlyA, experiments need to be done in the range of alkaline pH also. Acidic pH cannot be regarded as an absolute hostile environment for *Helicobacter pylori.* However the alkaline pH qualifies to be an outlandish environment for the survival of *Helicobacter pylori.* Hence it is necessary to understand the structural and functional aspect of TlyA in alkaline environment.

Recent studies have shown that *Helicobacter pylori* shows adaptation in response to high salt diet. In this scenario, it is tempting to analyse the structural and functional aspects of *H. pylori* TlyA under such an environment.

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