CHAPTER 3

RESULTS AND DISCUSSION

3.1 MOLECULAR BASIS OF COLD ADAPTATION OF LIPASE FROM Serratia marcescens

The first objective describes the cloning, expression, purification and enzyme kinetics of Serratia marcescens lipase LipA. A 1842 bp region of lipase was amplified from the genomic DNA of S.marcescens. The amplified region was cloned in to a prokaryotic expression vector, pRSET A and the authenticity of the clone was checked by PCR using different combinations of T7 primers and insert specific primers. The expression of recombinant lipase was obtained in GJ1158 strain of E.coli. The large-scale expression was optimized in GJ1158 strain of E.coli, since the induction can be achieved with 0.4 M NaCl. Recombinant LipA expressed as inclusion bodies was purified and solubilized. The solubilized LipA inclusion body along with the histidine tag was purified by IMAC. The IMAC purified LipA was refolded by pulse dilution. Purified and refolded LipA was assessed for lipase activity. The kinetics of the enzyme was measured at temperatures ranging from 4°C to 65 ºC. LipA revealed higher $K_M$ and $k_{cat}$ at 4ºC. Catalytic efficiency of LipA was maximum at 37 ºC.

3.1.1 Amplification and cloning of LipA gene in to T7 expression vector

Genomic DNA was isolated from wild type Serratia marcescens. The 1842 bp lipase gene was amplified using the wild type DNA as template (Figure 3.1a). The amplified LipA gene was purified by QIAquick PCR
purification column, digested with *BamHI* and *HindIII* restriction enzymes and ligated with *BamHI* and *HindIII* digested pRSET A vector. The ligation mixture was transformed in DH5α strain of *E.coli*. Ten transformants were selected for screening by PCR using insert specific primers. Figure 3.1b shows profile of PCR products. Out of ten transformants screened, only one transformant was positive for the presence of 1842 bp insert. This transformant (pLNC615) was used for further characterization.

**Figure 3.1 Amplification and cloning of 1842 bp LipA gene**

10 µl of the PCR products were loaded on 1.2 % agarose gel, stained with ethidium bromide (0.5 µg/ml) and observed in the Gel documentation unit.

a. **PCR amplification of LipA gene from Serratia marcescens**
   Lanes: M – 1Kb DNA molecular weight marker, S – amplified 1854 bp (1842 bp of LipA gene with histidine fusion tag)

b. **Screening of transformants by PCR**
   The insert in the pLNC615 was amplified by PCR using insert specific primers. Lanes: M – 1Kb DNA molecular weight marker, Pc – positive control (LipA gene from *S.marcescens*), 1 to 9 – negative transformants, 10 – positive transformant. The amplified product is indicated by an arrow.
3.1.2 Restriction digestion analysis of pLNC615

Plasmid (pLNC615) was prepared from the positive transformant and was subjected to restriction enzyme analysis with *Bam HI* and *Hind III* and compared with pRSET A restriction enzyme digestion profile. Single digestion with the above enzymes linearized pLNC615 to the size of approximately 4.75 Kb, whereas the pRSET A linearized to 2.9 Kb. When the plasmid, pLNC615 was subjected to double digestion with *Bam HI* and *Hind III*, the insert was released from the vector to the size of 1854 bp (Figure 3.2a), which confirmed the presence of the gene fragment in the vector.

3.1.3 Confirming the orientation of the insert in pLNC615

Orientation of the insert in the recombinant plasmid pLNC615 was checked by PCR using different combinations of T7 promoter and insert specific primers. The size of the amplicons, 2044 bp and 1944 bp obtained in the PCR confirmed the correct orientation of the insert in the pLNC615 (Figure 3.2b).
Figure 3.2 Confirmation of the insert and its orientation in the recombinant plasmid pLNC615

a. Restriction digestion analysis

2 µg of the recombinant plasmid (pLNC615) and pRSET A were digested with *BamH*I and *HindIII* and resolved on 1.2 % agarose gel. Lanes: M - 1 kb DNA molecular weight marker, 1 - double digested pLNC615.

b. Confirmation of orientation of the insert

The orientation of 1854 insert was confirmed by PCR using different combination of T7 promoter and insert specific primers. The PCR products were resolved on 1.2 % agarose gel. Lanes: M - 1 Kb DNA molecular weight marker, 1 - Insert specific primers, 2 - T7 promoter forward and insert reverse primers, 3 - Insert forward and T7 promoter reverse primers, 4 - Negative control.
3.1.4 Expression of recombinant protein in *E.coli*

Recombinant plasmid, pLNC615 was transformed into GJ1158 for high level expression. As lipase is an industrial enzyme, induction with a cheap and a non toxic source (NaCl) was preferred. So GJ1158 strain of *E.coli* was used for expression of the recombinant lipase from pLNC615. Initially, expression was tried in 3 ml of GYE with ampicillin by induction with 0.4 M NaCl. Leaky expression was observed in the uninduced culture. The salts present in the GYE medium were responsible for leaky expression. A polypeptide of approximately 65 kDa (Figure 3.3) molecular mass was expressed on induction. Time course analysis of expression increased for the first two hours after induction and remained constant thereafter. For large scale expression, a single colony was picked using a sterile loop and suspended in 50 ml of GYE medium. Culture was induced at $A_{600}$ 0.4 OD with 0.4M NaCl. The induced pRSET A was used as negative controls.

![Figure 3.3 Expression of recombinant LipA](image)

Total protein extracts from pLNC615 and pRSET A vector were solubilized in 1X SSB, resolved on 12% SDS-PAGE gel and stained with CBB dye. Lanes: M - protein molecular weight marker, 1 to 4 – Transformants expressing LipA at 1\textsuperscript{st}, 2\textsuperscript{nd}, 3\textsuperscript{rd} and 4\textsuperscript{th} hour, 5 - pRSET A Induced.
3.1.5 Purification of recombinant LipA

3.1.5.1 Purification of inclusion body

Recombinant LipA was expressed as inclusion body in GJ1158, since the levels of expression were very high. Less amount of protein was present in the soluble fraction. Inclusion bodies are considered to be formed by unspecific hydrophobic interactions between disorderly deposited polypeptides. Recent data suggest that these protein aggregates might be a reservoir of alternative conformational states, their formation being no less specific than the acquisition of the native-state structure (Salvador Ventura and Antonio Villaverde 2006). This implies that aggregation as inclusion bodies does not result in inactivation of enzymes. The expressed LipA inclusion bodies were prepared by cell lysis and high speed centrifugation. Inclusion body was purified by repeated washing with Tris-HCl and 1% DOC. Purified inclusion body was solubilized in 6 M urea.

3.1.5.2 Purification of LipA using IMAC

Solubilized pure inclusion bodies were used for purification on IMAC columns. An imidazole based elution was used to obtain the purified protein. Higher concentrations of imidazole were used, as the standard low concentration did not result in elution of the protein. Optimization of IMAC for high-grade purification was attempted by changing different parameters and increasing the amount of purified inclusion body. Since purified inclusion bodies were used as load, no protein was observed in flow through. Recombinant LipA was eluted in 1 M fraction of imidazole (Figure 3.4a). Purified protein was confirmed by Western blotting with anti-his monoclonal antibody as the primary antibody (Figure 3.4b).
3.1.5.3 Refolding of purified LipA

Purified LipA was refolded by pulse renaturation process. 1ml of sample containing 50 µg/ml of purified LipA was added drop wise to 10 ml of renaturation buffer (50mM Tris pH 8.0, 20mM CaCl$_2$) at 4ºC. Renaturation buffer was mixed continuously on a magnetic stirrer to avoid formation of aggregates. The success of this process is based on the fact that once a small amount of denatured protein is refolded into the native form, it does not form an aggregate with the unfolded protein.

Figure 3.4 Purification of recombinant LipA by IMAC

(a) **Purification by IMAC**

The recombinant LipA was eluted at 1 M imidazole concentration from Ni$^{2+}$ bound sepharose column. The wells were loaded with 20 µl volume of the following on 12% SDS-PAGE. Lanes: M – protein molecular weight marker, 1 – total protein extract from pLNC615 (50 µg), 2 – soluble fraction of pLNC615, 3 – Inclusion bodies of pLNC615, 4 – purified inclusion bodies used for loading the column, 5 – Flow through and 6 – 1 M imidazole eluent (5 µg).

(b) **Conformation of purified recombinant LipA by Western-blotting**

10 µg of purified recombinant LipA from pLNC615 was resolved on 12% SDS-PAGE gel, was transferred on to a nitrocellulose membrane, and was probed with anti-his monclonal antibody. Lanes: 1-protein molecular weight marker, 2-purified LipA.
3.1.6 Functional characterization of LipA

To ensure that bioactive protein was formed during refolding of recombinant LipA, activity was measured at standard assay conditions (Table 3.1).

Table 3.1 Activity of refolded LipA at standard assay conditions

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Lipase Activity U/ml</th>
<th>Protein concentration mg/l</th>
<th>Specific Activity U/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>LipA</td>
<td>9.18</td>
<td>442.3</td>
<td>20.77</td>
</tr>
</tbody>
</table>

Higher specific activity displayed by refolded LipA at standard assay conditions proved that LipA was refolded to a bioactive three dimensional conformation in the presence of calcium in the refolding buffer.

Enzyme kinetics was measured at different temperatures in order to assess the effect of these parameters on activity. Aliquots of refolded LipA from the same sample were used for all the assays. Substrate concentration (4-nitrophenolate) used for the assay ranges from 0.5mM to 2.5mM at each temperature. Values used for Lineweaver-Burk plot were average of three experiments. Reciprocal of lipase units and substrate concentration were used to plot the Lineweaver-Burk plot. $K_m$ of the enzyme was obtained from X-intercept value of the LB plot and $V_{max}$ was obtained from Y-intercept value. Turnover number and catalytic efficiency of LipA was calculated from the obtained $K_m$ and $V_{max}$ values for each temperature.
Figure 3.5 (a) Kinetics of LipA at 4 °C and (b) Kinetics of LipA at 15°C
Figure 3.6 (a) Kinetics of LipA at 25°C and (b) kinetics of LipA at 37°C
Figure 3.7 (a) Kinetics of LipA at 45°C and (b) Kinetics of LipA at 55°C
The kinetics parameters determined at various temperatures for the recombinant LipA are illustrated in figures 3.5, 3.6, 3.7 and 3.8 and summarized in table 3.2. It has been reported that psychrotolerant enzymes have higher $K_M$ values in order to maximize the overall reaction rate. Similar functional characterization was observed with LipA. LipA displayed highest $K_M$ and $k_{cat}$ values at 4°C in a temperature range of 4°C to 65°C. The apparent maximum catalytic efficiency of LipA was found to be at 37°C. However, 44% of maximum catalytic efficiency was still recorded at 4°C. High $K_M$ and $k_{cat}$ values were responsible for lowering the catalytic efficiency at 4°C. These results clearly indicate that substrate affinity is the rate limiting factor at lower temperatures for LipA. Catalytic constants of the enzyme were drastically reduced above 45°C, rendering the protein to be thermally inactive.
This molecular strategy of LipA clearly displays the evolutionary adaptation of mesophilic enzymes to lower temperature.

**Table 3.2 Kinetic constants of LipA**

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat} / K_M$ (s$^{-1}$, µM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>10</td>
<td>113.05</td>
<td>11.30</td>
</tr>
<tr>
<td>15</td>
<td>4.03</td>
<td>59.5</td>
<td>14.87</td>
</tr>
<tr>
<td>25</td>
<td>2.84</td>
<td>50.24</td>
<td>17.69</td>
</tr>
<tr>
<td>37</td>
<td>3.16</td>
<td>80.75</td>
<td>25.55</td>
</tr>
<tr>
<td>45</td>
<td>3.35</td>
<td>64.59</td>
<td>19.28</td>
</tr>
<tr>
<td>55</td>
<td>0.82</td>
<td>7.293</td>
<td>8.829</td>
</tr>
<tr>
<td>65</td>
<td>0.53</td>
<td>0.127</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Kinetic constants were determined from initial rates at different concentrations of 4-nitrophenolate over the range of 0.5mM – 2.5mM in undecane. The values are averages of three experiments and standard errors are less than 10%.

3.1.7 Thermal stability of recombinant LipA

Recombinant pure protein was held at temperatures ranging from 45°C to 85°C for 15 minutes. Heating time did not include the time to attain the required temperature. Assay was carried out for each treatment to obtain lipase stability. The recombinant LipA showed 70% stability (Figure 3.9) up till 60°C, after which there was a gradual decrease in the activity with increase in temperature, but the least residual activity was at 85°C. It was completely inactivated at 90°C.
Figure 3.9 Thermal Stability of recombinant LipA

LipA from *Serratia marcescens* is the first enzyme of its family to have been studied for cold adaptation. LipA being an isolate of psychrotrophic organism, the optimal temperature for activity was at 37°C and also showed 44% maximum catalytic efficiency at 4°C. Cold-active enzymes are frequently thermolabile, since they have to be flexible enough to be active at a low energy cost (Feller et al 1997). Cold adapted enzymes counteract the inhibitory effect of low temperature on activity by reducing the temperature dependence of the reaction rate (low activation enthalpy). As this low activation enthalpy reflects the smaller number of enthalpy-driven interactions that have to be broken to reach the activated transition state, it has been proposed that the activity of a psychrophilic enzyme is heat labile, as these interactions also contribute to the active site architecture (Fields et al 1998). However, LipA was 70% stable till 60°C. The stability of LipA at higher temperatures could be dependent on the Ca²⁺ ions bound to the nonapeptide in the C-terminal domain. The involvement of calcium binding domains in stability is particularly very clear in *P.flourescens* lipase. In mutant of *P.fluorescens* lipase, on reduction of RTX calcium binding repeated motifs to
less than five, a β-roll structure is not formed or poorly formed leading to a lesser stable protein (Kwon et al 2002).

The characteristics of LipA to be active at lower temperatures could be attributed to localized flexibility. The N-terminal domain of LipA possesses the catalytic triad with the mobile lids required for activity, and the C-terminal domain possesses the RTX binding motifs along with the secretion signal. We speculate that the amino terminal domain as the heat labile domain providing the flexibility to be catalytically active at lower temperature and carboxy domain behaves as the heat stable domain providing stability to whole protein.

Analysis of kinetic parameters of LipA at different temperatures proved highly significant in terms of functional adaptation to cold. Ideally, adapting an enzyme to cold would mean optimizing both $K_M$ and $k_{cat}$. Such a trend was noted for enzymes from Antarctic or Arctic fishes and some bacterial enzymes (Bentahir et al 2000). However, a survey of cold-adapted enzymes showed that optimization of the $k_{cat} / K_M$ ratio is far from universal (Glansdorff et al 2002); for instance, markedly high $K_M$ values have been reported for psychrophilic glutamate dehydrogenase (Fraia et al 2000), citrate synthase (Gerike et al 1997), aspartate carboxamoyltransferase (Sun et al 1998), aspartate aminotransferase (Birolo et al 2000), triosephosphate isomerase (Alvarez et al 1998), DNA ligase (Georlette et al 2000), elongation factor Tu (Masullo et al 2000), subtilisin (Narinx et al 1997), xylanase (Collins et al 2002), and alpha-amylase (D’Amico et al 2001). LipA belongs to this group, as shown by the high $K_M$ value at 4°C, indicating a low substrate binding affinity.

Therefore, it appears that numerous cold active enzymes improve $k_{cat}$ values at low temperatures at the expense of $K_M$ values. Several aspects
seem to be involved in this adaptive strategy. (i) From the kinetic and thermodynamic theories, it is well known that weak substrate binding is catalytically advantageous (Fresht et al 1985); indeed, the ground-state enzyme-substrate complex falls in a less deep energy pit, therefore reducing the energy barrier (and increasing $k_{cat}$) for the reaction (Feller et al 1997). (ii) The large activation entropy variation in psychrophilic enzymes suggests large conformational movements between a loose active site in the free state and the tightly bound transition state (Fields et al 1998); such loosely structured active sites should bind a substrate weakly. (iii) According to the folding-funnel hypothesis and to achieve the flexibility required for an enzyme to function efficiently at a lower temperature, a larger number of conformational states must be available to the enzyme; therefore, the enzyme may exhibit a higher $K_M$ and, possibly, a lower affinity for the substrate(s) of the reaction (Lonhienne et al 2000).

3.2  BI-FUNCTIONAL NATURE OF LipA

The second objective describes the bi-functional nature displayed by LipA from *Serratia marcescens*. Amino acid sequence analysis of LipA C-terminal domain showed a significant degree of homology with E.coli $\alpha$-hemolysin. Recombinant protein expressed by clone of LipA (pLNC615) was analyzed for the presence of hemolytic activity on spectrophotometric erythrocyte cleavage of erythrocytes. LipA displayed hemolytic activity along with lipase activity rendering it to be a bi-functional protein.

3.2.1  Amino acid sequence analysis of LipA for bi-functional activity

BLAST analysis of the amino acid sequence of LipA showed homology with RTX toxins and with Family 1.3 lipases (Appendix 1). Results of BLASTP revealed significant degree of homology between carboxy-
terminal part of LipA and *E.coli* alpha hemolysin and adenylate cyclase from *Bordetella pertussis*. The score and e-value results shown by BLASTP analysis of LipA are given in Appendix 1. Pairwise alignment was performed using EMBOSS, between carboxy-terminal part of LipA and HlyA of *E.coli*, it showed an identity of 26.3% and similarity of 35.4% (Figure 3.10). Since the sequences are extensively related in the nonapeptide region, it could be assumed that they are also functionally related and LipA may exhibit hemolytic activity along with lipase activity.

<table>
<thead>
<tr>
<th>Lip</th>
<th>13 SLTDKDSTIIVSNLSNVTRGNTW---VEDLNRAETHSGPTFIIGSDGN</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLYAC_ECOLX</td>
<td>708NLTEFDNLVSVEELIGTRADKFGSKFADIFHGAD---GDDHIEGNDGN</td>
</tr>
<tr>
<td>Lip</td>
<td>59 DLIKGGKGNYLEGRTGDIFRDAGGYNLIAAGKGN1FFDTQKLKNTEV</td>
</tr>
<tr>
<td>HLYAC_ECOLX</td>
<td>755DRLYDGKNDTSLGNDQLYGDDGKNDKLLGANN-------------------</td>
</tr>
<tr>
<td>Lip</td>
<td>109AYDGNTLRLDKGTADDISTLRKSMTWLFNKEVDFQVTVAGLKS</td>
</tr>
<tr>
<td>HLYAC_ECOLX</td>
<td>792------YLN----------------------------------------</td>
</tr>
<tr>
<td>Lip</td>
<td>159DSGLKAYAATGDDVQLQARSHDA---WLFGRANNDTLDHAGGNLTF</td>
</tr>
<tr>
<td>HLYAC_ECOLX</td>
<td>794-------------NGGDGDELQVQGNSLAKNVSGLGKNDKLYGEGADL-L</td>
</tr>
<tr>
<td>Lip</td>
<td>206VGSGDDILKGVCNNTF--------LFSGDGRQLYGFRNASDKLVF</td>
</tr>
<tr>
<td>HLYAC_ECOLX</td>
<td>833DGSGNDLKG-GYNDIYRLSGLYHHIIDDDGK------DKLSL</td>
</tr>
<tr>
<td>Lip</td>
<td>246IGITASGNIIRDYA-TQONDDLVLAFGHSQVTLIG</td>
</tr>
<tr>
<td>HLYAC_ECOLX</td>
<td>874-----ADIDFRDVAFRREGNDLIMYKAEGNVLISIG</td>
</tr>
</tbody>
</table>

**Figure 3.10** Pair-wise alignment of LipA with HlyA

Pairwise alignment between C-terminal domains of LipA and HlyA in EMBOSS-water, showing a similarity of 35.4%. Lip – C-terminal domain of LipA from *S.marcescens*, HLYAC_ECOLX – C-terminal domain of HlyA from *E.coli*
3.2.2 Hemolytic activity of LipA

The amino acid sequence analysis of LipA results indicated that LipA was homologous with hemolysin, archetype of RTX toxins. Therefore, purified and refolded recombinant LipA was subjected to hemolytic assay in the presence of calcium. LipA encoded by pLNC615 caused hemolysis of 74% of whole human blood on spectrophotometric measurement of erythrocyte cleavage. This result indicates that in S.marcescens, the LipA by itself, displayed hemolytic activity in the presence of calcium ion. These data also demonstrate the importance of calcium ions binding to RTX region in hemolytic activity of LipA.

LipA is a member of the RTX toxin family (Meier et al 2007), which also includes the E.coli α-hemolysin (HlyA) and Bordetella pertussis cyclolysin, where the repeats come in blocks separated by linker sequences. A major characteristic of these toxins is their capacity to lyse Red blood cells (hemolytic activity). Hemolytic activity endowed by hemolysin, includes two main steps, namely reversible binding (adsorption) and irreversible insertion (Bakas et al 1996). Binding occurs via receptors (Cortajarena et al 2003) although the latter is not essential, since the toxin N-terminal amphipathic helix domain adsorb onto the membrane surface, become inserted in the bilayer, and finally disrupt the membrane permeability barrier (Hyland et al 2001). In LipA hemolysis occurs in the absence of receptors as the amino acid sequence of LipA lacks the conserved regions near the RTX domain, to bind with α-glycophorin receptors on the host membrane. In support of lipA hemolysin activity we presume that the N-terminal domain, characterized by amphipathic helices in an alpha-beta fold, interacts with hydrophobic matrix releasing fatty acids required for pore formation, causing subsequent loss of permeability barrier in the host membrane, in the presence of calcium. In this
way, the hemolytic activity of LipA would be complemented by lipase activity.

This kind of vesicle leakage or cell lysis requires calcium binding to nona-peptide repeats prior to interaction with membranes (Ostolaza et al 1995). Analysis of the mutant proteins of HlyA suggests that the repetitive sequences are essential for activity (Felmlee et al 1988). In adenylate cyclase, the adjacent polypeptide sequences are essential for the folding and calcium responsiveness of the RTX module (Bauche et al 2006). In addition, Ca\(^{2+}\) binding appears to expose hydrophobic patches on the protein surface, facilitating irreversible insertion into membranes and cellular reactions are triggered by uncontrolled fluxes of ions, particularly calcium (Valeva et al 2005). These observations suggest that LipA, of *S.marcescens* in the absence of membrane binding receptor, binds nonspecifically to lipid bilayers and cellular reactions are triggered by uncontrolled fluxes of Ca\(^{2+}\) ions, through the pores.

The coexistence of lipase and hemolytic activity in LipA, rendering it to be a bi-functional protein enhances the virulence nature of LipA. LipA acts as an important virulence factor in *Serratia marcescens*. On a comparative analysis of *Serratia* natural isolates to clinical strains, 95% of potential virulent strains secrete LipA (Fanczek et al 1986). The most prominent virulent role played by extracellular lipases is digestion of lipids for acquisition of nutrients. This process releases free fatty acids which help in cell to host adhesion. In some cases, lipase triggers the immune system to produce anti-lipase IgG antibodies. Hemolysins are membrane targeted toxins proven to be influential on hemorrhagic intestinal disease, whooping cough and urinary tract infections. Hemolysin attacks erythrocytes, granulocytes, monocytes, endothelial cells and renal epithelial cells. Hemolysins alter the
membrane permeability of host cells, causing lysis and death. Lysis of erythrocytes provides bacteria with iron.

3.3 FUNCTIONAL CHARACTERIZATION OF DOMAINS OF LipA

The third objective of the work was to assess whether the amino terminal domain having the catalytic triad with the mobile lids required for lipase activity, could function independently as a bioactive protein and retaining its thermostable nature along with cold adaptation. The work was done, also to identify if the amino-terminal domain (aa 1-320) could have a structural role in the hemolytic activity, or whether the carboxyl-terminal part of LipA could achieve its active conformation in the absence of the amino-terminal moiety.

We constructed two truncated LipA gene, N-terminal domain encoding 1-320aa of LipA and C-terminal domain encoding the last 295 aa of LipA, harbored by plasmids (pLN320 and pLC295) that replicate in E.coli. 957 bp N-terminal domain and 885 bp C-terminal domain of LipA was amplified from pLNC615 parent clone. The amplified region was cloned in to a prokaryotic expression vector, pRSET A and the authenticity of the clone was checked by PCR using different combinations of T7 primers and insert specific primers. The expression of recombinant lipase was obtained in GJ1158 strain of E.coli. The large-scale expression was optimized in GJ1158 strain of E.coli, since the induction can be achieved with 0.4 M NaCl. Recombinant mutant LipA proteins expressed as inclusion bodies was purified and solubilized. The solubilized amino-terminal and carboxy-terminal mutant inclusion bodies along with the histidine tag were purified by IMAC. The IMAC purified mutant LipA proteins were refolded by pulse renaturation. Purified and refolded mutant LipA proteins were assessed for
lipase and hemolytic activity. Protein expressed by N-terminal domain of LipA showed very less lipase activity and thermostability nature of the native protein was completely lost in the mutants. There was complete loss of hemolytic activity in N- and C-terminal domains. C-terminal domain did not display lipase activity.

3.3.1 Amplification and cloning of amino-terminal LipA gene and carboxy-terminal LipA gene in to T7 expression vector

The 957 bp N-terminal lipase gene and 885 C-terminal lipase genes were amplified using the pLNC615 clone as template (Figure 3.11a and b). The amplified mutant genes were purified by QIAquick PCR purification column, digested with BamHI and HindIII restriction enzymes and ligated with BamHI and HindIII digested pRSET A vector. The ligation mixture was transformed in DH5α strain of E.coli. Ten transformants for each clone were selected for screening by PCR using insert specific primers. Figure 3.12a and b shows profile of PCR products. Out of ten transformants screened for N-terminal lipase clone, only one transformants was positive for the presence of 957 bp insert (pLN320). In C-terminal lipase clone, all the screened transformants were positive. The single positive transformant of N-terminal lipase clone and one of the positive transformant of C-terminal clone (pLC295) were used for further characterization.
Figure 3.11 Amplification LipA mutants

10 µl of the PCR products were loaded on 1.2 % agarose gel, stained with ethidium bromide (0.5 µg/ml) and observed in the Gel documentation unit.

a. PCR amplification of N-terminal domain of LipA gene from *Serratia marcescens*
   Lanes: M – 1Kb DNA molecular weight marker, S – amplified 969 bp (957 bp of N-terminal domain of LipA gene with histidine fusion tag).

b. PCR amplification of C-terminal domain of LipA gene from *Serratia marcescens*
   Lanes: M – 1Kb DNA molecular weight marker, S – amplified 897 bp (885 bp of C-terminal domain of LipA gene with histidine fusion tag).
Figure 3.12  Cloning of LipA mutant genes into pRSET A vector

(a) Screening of N-terminal LipA transformant by PCR
The insert in the pLN320 was amplified by PCR using insert specific primers. Lanes: 1, 2, 4 to 10 – negative transformants, 3 – positive transformant. The amplified product is indicated by an arrow.

(b) Screening of C-terminal LipA transformant by PCR
The insert in the pLC295 was amplified by PCR using T7 forward and insert specific reverse primers. Lanes: M – 1Kb DNA molecular weight marker, 1 to 10 – positive transformants. The amplified product is indicated by an arrow.
3.3.2 **Restriction digestion analysis of LipA mutants**

Plasmids (pLN320 and pLC295) were prepared from the positive transformants and were subjected to restriction enzyme analysis with *Bam HI* and *Hind III* and compared with pRSET A restriction enzyme digestion profile. Single digestion with the above enzymes linearized pLN320 to the size of approximately 3.85 Kb and pLC295 to 3.75 Kb, whereas the pRSET A linearized to 2.9 Kb. When the plasmids, pLN320 and pLC295 were subjected to double digestion with *Bam HI* and *HindIII*, the insert was released from the vector to the size of 957 bp for N-terminal LipA and 885 bp for C-terminal LipA (Figure 3.13), which confirmed the presence of the gene fragment in the vector.

![Restriction digestion analysis](image)

**Figure 3.13 Confirmation of the insert by restriction digestion analysis**

2 μg of the recombinant plasmids (pLN320 and pLC295) and pRSET A were digested with *BamHI* and *HindIII* and resolved on 1.2 % agarose gel. Lanes: M- 1 kb DNA molecular weight marker, 1 – double digested pLN320, 2 – double digested pLC295
3.3.3 Expression of recombinant mutant proteins in *E.coli*

Each recombinant plasmid (pLN320 and pLC295) was transformed independently into GJ1158 for high level expression. As both clones encoded mutants of LipA, induction procedure similar to parent clone (pLNC615) was followed. Mutant clones were also expressed as inclusion bodies. Polypeptide of approximately 35.2 kDa (Figure 3.14) was synthesized by pLN320 and 33.5 kDa (Figure 3.15) molecular mass was expressed by pLC295 on induction. Time course analysis of expression of pLN320 remains constant from the first hour of induction because of the leaky expression on addition of salts to the GYE medium. pLC295 time course analysis showed that the expression was low for the first 2 hours and increased to maximum in the 3rd hour. For large scale expression, a single colony was picked using a sterile loop and suspended in 50 ml of GYE medium. Culture was induced at $A_{600}$ 0.4 OD with 0.4M NaCl. The induced pRSET A was used as negative controls.

![Expression of N-terminal domain of LipA](image)

**Figure 3.14 Expression of N-terminal domain of LipA**

Total protein extracts from pLN320 and pRSET A vector were solubilized in 1X SSB, resolved on 12% SDS-PAGE gel and stained with CBB dye. Lanes: M - protein molecular weight marker, 1 to 3 – Transformants expressing N-terminal domain of LipA at first, second and third hour, 4 - pRSET A– Induced.
Figure 3.15 Expression of C-terminal domain of LipA

Total protein extracts from pLC295 and pRSET A vector were solubilized in 1X SSB, resolved on 12% SDS-PAGE gel and stained with CBB dye. Lanes: M - protein molecular weight marker, 1,2 - Transformants expressing C-terminal domain of LipA for the first and second hour, 3 - pRSET A– Induced, 4 – Transformant expressing C-terminal domain of LipA at the third hour.

3.3.4 Purification of recombinant mutant LipA proteins

Recombinant mutant LipA proteins synthesized by pLN320 and pLC295 were expressed as inclusion bodies in GJ1158. Since the levels of expression were very high, only traces of protein were present in the soluble fraction in both cases (Figure 3.16 and 3.17). Expressed inclusion bodies of the mutant clones were prepared by cell lysis and repeated washing with Tris-HCL and 1% sodium deoxy cholate solution. Purified inclusion bodies of each clone (pLN320 and pLC295) were solubilized in 6M urea.

Solubilized pure inclusion bodies of both clones were further purified on IMAC columns. An imidazole based elution was used to obtain the purified proteins. Concentration of imidazole similar to template protein
(pLNC615) was used for purification of the mutants. As purified inclusion bodies were used as load, no protein was observed in flow through even in the case of mutant proteins. Both recombinant mutant proteins of LipA were eluted in the 1 M fraction of imidazole (Figure 3.16 and 3.17) analogous to pLNC615. Purified mutant proteins were confirmed by Western blotting with anti-his monoclonal antibody as the primary antibody (Figure 3.18).

Purified N-terminal LipA domain and C-terminal LipA domain recombinant proteins were refolded by pulse renaturation processes similar to recombinant LipA.

![Figure 3.16 Purification of N-terminal LipA by IMAC](image)

The recombinant N-terminal LipA was eluted at 1 M imidazole concentration from Ni$^{2+}$ bound sepharose column. The wells were loaded with 20 μl volume of the following on 12% SDS-PAGE. Lanes: 
M – protein molecular weight marker, 1 – total protein extract from pLN320 (50 μg), 2 – soluble fraction of pLNC615, 3 – Inclusion bodies of pLNC615, 4 – purified inclusion bodies used for loading the column, 5 – Flow through and 6 – 1 M imidazole eluent (5 μg).
Figure 3.17  Purification of C-terminal LipA by IMAC

The recombinant C-terminal LipA was also eluted at 1 M imidazole concentration from Ni$^{2+}$ bound sepharose column. The wells were loaded with 20 µl volume of the following on 12% SDS-PAGE. Lanes: M – protein molecular weight marker, 1 – total protein extract from pLN320 (50 µg), 2 – soluble fraction of pLNC615, 3 – Inclusion bodies of pLNC615, 4 – purified inclusion bodies used for loading the column, 5 – Flow through and 6 – 1 M imidazole eluent (5 µg).
Figure 3.18 Western-blotting of LipA mutants

(a) **Analysis of IMAC purified N-terminal domain of LipA** 10µg of purified recombinant N-terminal domain of LipA from pLN320 was resolved on 12% SDS-PAGE gel, was transferred on to a nitrocellulose membrane, and was probed with anti-his monoclonal antibody. Lanes: S- purified N-terminal domain of LipA, M- protein molecular weight marker.

(b) **Analysis of IMAC purified C-terminal domain of LipA** 10µg of purified recombinant C-terminal domain of LipA from pLC295 was resolved on 12% SDS-PAGE gel, was transferred on to a nitrocellulose membrane, and was probed with anti-his monoclonal antibody. Lanes: M-protein molecular weight marker, 1-purified C-terminal domain of LipA.

3.3.5 Functional characterization of N-terminal and C-terminal domains of LipA

N-terminal and C-terminal domains are fragments of LipA, so it was assumed that using an analogous procedure for refolding would also result in bioactive proteins for the mutants. Lipase assay was done for both truncated proteins in a temperature range of 4°C to 37°C. C-terminal domain displayed no lipase activity at all temperatures, proving our assumption of N-terminal domain to be the catalytic domain as true. Lipase activity of the
truncated catalytic domain of LipA showed closely similar values at all temperatures (Table 3.3). As the concentration of the mutant protein chosen for analysis was uniform, not much difference was observed in the specific activity at various temperatures. The mutant pLN320 encoding the N-terminal domain was not stable above 45ºC. The specific activity of N-terminal LipA was 12% relative to its full length LipA at 4ºC (Table 3.3).

. Table 3.3 Lipase activity of refolded N-terminal LipA mutant protein

<table>
<thead>
<tr>
<th>Temperature ºC</th>
<th>Lipase Activity U/ml</th>
<th>Protein concentration mg/l</th>
<th>Specific Activity U/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.756</td>
<td>362.8</td>
<td>2.1</td>
</tr>
<tr>
<td>15</td>
<td>0.756</td>
<td>362.8</td>
<td>2.1</td>
</tr>
<tr>
<td>25</td>
<td>0.724</td>
<td>362.8</td>
<td>2.0</td>
</tr>
<tr>
<td>37</td>
<td>0.844</td>
<td>362.8</td>
<td>2.3</td>
</tr>
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</table>

Lipase activity of mutant N-terminal LipA was done using standard assay conditions at temperatures ranging from 4ºC to 37ºC. 1mM concentration of 4-nitrophenolate was used as the substrate.
3.3.6 Kinetics N-terminal domain of LipA

Figure 3.19 Kinetics of N-terminal domain of LipA at 4°C (1/V₀ - s.µM⁻¹, 1/S₀ - µM⁻¹)

Figure 3.20 Kinetics of N-terminal domain of LipA at 15°C (1/V₀ - s.µM⁻¹, 1/S₀ - µM⁻¹)
Figure 3.21 Kinetics of N-terminal domain of LipA at 25°C (1/V₀ - s.µM⁻¹, 1/S₀ - µM⁻¹)

Figure 3.22 Kinetics of N-terminal domain of LipA at 37°C (1/V₀ - s.µM⁻¹, 1/S₀ - µM⁻¹)
Cold adaptation and thermal inactivation of the recombinant N-terminal LipA mutant was analyzed by measuring lipase activity at different temperatures (Fig 1, 2, 3, 4) (table 1). The pattern of kinetic properties of mutant LipA was identical to native LipA. The N-terminal mutant LipA displayed highest Km and kcat at 4C in a temperature range of 4 C to 37 C similar to native LipA. Furthermore, mutant N-terminal LipA was completely inactivated above 45C, rendering the protein to be thermally unstable. Thermolabile nature at low temperature is a clear evidence of the cold adaptive nature displayed by N-terminal domain of LipA.

The catalytic efficiency of mutant amino-terminal LipA was 20-25% relative to native LipA protein at different temperatures (table 1). This implies that the mutant N-terminal domain is only partially folded to an active conformation in the absence of the carboxy domain. It has been proposed that the parallel β-roll structure act as an intramolecular chaperone that keeps the polypeptide unfolded in the cytoplasm where Ca²⁺ concentrations are less, although in the external medium with Ca²⁺ concentrations in mM range the glycine repeats would fold spontaneously and provides a nucleus for the folding of the entire polypeptide chain (Meier et al 2007). Studies on the homologous *Pseudomonas* sp. MIS38 lipase have shown that partial or complete knock-out of the tandem repeats leads to strongly reduced protein and secretion levels. Furthermore, knock-out of all repeats resulted in a virtually inactive enzyme (Kwon et al 2002). The intracellular stability is also

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>$K_M$ (µM)</th>
<th>$K_{cat}$ (S⁻¹)</th>
<th>$K_{cat}/K_M$ (S⁻¹. µM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.23</td>
<td>4.35</td>
<td>10.2</td>
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</tr>
<tr>
<td>0.57</td>
<td>1.75</td>
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<td>1.00</td>
<td>4.45</td>
<td>4.56</td>
</tr>
<tr>
<td>1.02</td>
<td>0.98</td>
<td>5.3</td>
<td>5.4</td>
</tr>
</tbody>
</table>

*Table 3.4 Kinetic constants of mutant N-terminal domain of LipA*
affected, being about 600 times lower than that of the complete protein. The second set of tandem repeats pack laterally against the first β-roll domain. This appears to be a general feature of all RTX proteins possessing more than eight residue motifs, e.g. *E.coli* hemolysin or *Bordetella pertussis* cyclolysin. In this way, the β-roll domains become integral parts of the whole structure and can act as folding nuclei, stabilizing different parts of the catalytic N-terminal domain. The reduction in activity of mutant LipA due to improper folding confirms the role of β-roll structure as an intramolecular chaperone.

Thermal instability displayed by N-terminal LipA domain also reveals the overall stability governed by the carboxy domain on native LipA. No lipase activity was recorded by the C-terminal LipA mutant. Difference in thermal stability recorded by the N-terminal mutant protein in relative to native LipA reveals that the native LipA from *S.marcescens* is composed of a heat labile domain and a heat stable domain.

Cold enzymes are categorized by a low conformational stability, that even multidomain proteins display a reduced stability of all calorimetric units and that they have evolved toward the lowest available stability of the native state (Gerday et al 1999). The results obtained for LipA and its mutants demonstrate that this evolution can affect only one particular domain of the molecule. The concept of stabilizing domains has emerged from studies of proteins such as xylanases (Fontes et al 1995), in which the increased stability of one domain promotes the stability of the whole molecule. So we consider that the catalytic heat labile N-terminal domain of LipA as destabilizing domain, providing the required flexibility around the active site in order to increase the catalytic rate at low temperature. High flexibility of the mutant amino terminal LipA molecule is accompanied by broader distribution of conformation states, leading to a poor ligand binding and high Km values.
The mutant plasmids pLN320 and pLC295 encoded proteins were subjected to erythrocyte cleavage. Both displayed no detectable hemolytic activity in the presence of calcium when compared to full length LipA. We conclude that both N-terminal and C-terminal domain are nonlytic as separate domains.

Lipase activity of amino-terminal LipA mutant is considerably reduced at lower temperature. This implies that the amino terminal domain is not folded to an active conformation in the absence of C-terminal domain. It has been proposed that the parallel β-roll structure act as an intramolecular chaperone that keeps the polypeptide unfolded in the cytoplasm where Ca\(^{2+}\) concentrations are less, although in the external medium with Ca\(^{2+}\) concentrations in mM range the glycine repeats would fold spontaneously and provides a nucleus for the folding of the entire polypeptide chain (Meier et al 2007). Studies on the homologous *Pseudomonas* sp. MIS38 lipase have shown that partial or complete knock-out of the tandem repeats leads to strongly reduced protein and secretion levels. Furthermore, knock-out of all repeats resulted in a virtually inactive enzyme (Kwon et al 2002). The intracellular stability is also affected, being about 600 times lower than that of the complete protein. The second set of tandem repeats pack laterally against the first β-roll domain. This appears to be general feature of all RTX proteins possessing more than eight residue motifs, e.g. *E.coli* hemolysin or *Bordetella pertussis* cyclolysin. In this way, the β-roll domains become integral parts of the whole structure and can act as a folding nuclei, stabilizing different parts of the catalytic N-terminal domain. Larger the N-terminal domain, more the β-rolls are needed.

Our results suggest that both domains, within the LipA are required for hemolysis: deletion of either of the domains resulted in a complete loss of
hemolytic activity. The fact that lipA mutants, pLN320 and pLC295 are non-lytic, preclude a role for the domains to be active as a separate protein. These observations suggest that our interpretation of the synergistic role played by two domains to be hemolytic is true.

Thus we propose that both the N-terminal, alpha-beta fold domain and the C-terminal calcium binding domain of lipA are directly but differently involved in hydrolysis of triglycerides and hemolysis of red blood cells. The carboxy domain being responsible for stability and adsorption on to the surface of red blood cells and the amino terminal domain for flexibility and interaction with hydrophobic matrix of red blood cells.