CHAPTER 6

Purification of Staphylococcus arlettae lipase
ABSTRACT

An extracellular thermostable lipase was purified to homogeneity by using anion exchange chromatography and gel-filtration chromatography using DAEF-Sepharose and Sephacryl S-200 columns, respectively, with 27 fold purification and 32.5 U/mg specific activity. The purified enzyme showed a prominent single band on SDS–PAGE. The molecular weight of the purified enzyme was estimated to be 45 kDa. Based on matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometer (MALDI-TOF-MS) identified an amino acid sequence which shared similarity with α/β hydrolase fold gj427702968 Cyanobium gracile PCC 6307.

6.1 INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are one of the most important classes of hydrolytic enzymes that catalyse both the hydrolysis and the synthesis of esters. Moreover, bacterial lipases are preferential enzymes for a wide range of industrial reactions due to its catalytic activity in aqueous and non-aqueous reactions. Only about 2% of world’s microorganisms have been tested as enzyme sources until 2006, and lipases from different sources have large variations in enzymatic activity, fatty acid specificity, optimal temperature, and pH (Hasan et al., 2006). Lipases with molecular weight range of 19–60 kDa belong to the α/β hydrolase family. The active site is formed by a catalytic triad of Ser, Asp/Glu and His. The active site of lipase is covered by a lid-like α-helical structure. The lid moves away upon binding to a lipid interface, causing the active site of lipase fully accessible, enhancing hydrophobic interaction between the enzyme and lipid surface (Jaeger and Reetz, 1998). Extracellular lipases have been proven to be efficient and selective biocatalysts in many industrial applications such as biosensors, pharmaceuticals, foods, cosmetics, detergents (Pandey et al., 1999). Lipases differ from one another by size, substrate specificity, stability profile, and activity in the presence of various activators and inhibitors. Given the importance of lipases in various industrial applications, there is much interest in isolating novel enzymes from unique environmental niches. Although lipases can be obtained from plants and animals, microbial lipases possess useful features such as high
yield and low production cost, diversity in catalytic activities, amenability to genetic manipulation, stability in organic solvents and broad substrate specificity (Shu et al., 2010). While a large number of different lipases have been discovered and commercialized (Hasan et al., 2006), new lipases with better characteristics are desirable, such as high activity and stability in non-aqueous media, (Xiao et al., 2009 and Bisen et al., 2010), and stability under alkaline conditions and in the presence of surfactants for use in detergent formulations (Hasan et al., 2010). Lipases have been purified from animal, plant, fungal and bacterial sources using variety of methods involving ammonium sulphate precipitation, ion exchange chromatography followed by gel filtration. Since industrial processes are commonly carried out under harsh conditions, it would be of great importance to obtain lipases which retain their optimal activity at extremes of temperature, pH, different concentrations of salts and in the presence of organic compounds normally used in the industrial reactions as solvents (Mellado et al., 2005). In this sense, the lipases isolated from extreme microorganisms constitute an excellent alternative in the industrial processes (Pikuta et al., 2007). Furthermore, the purified peptides were applied to the matrix-assisted laser desorption ionization- time of flight tandem mass spectrometer (MALDI-TOF/TOF MS) to determine their molecular mass and their amino acid sequence.

6.2 MATERIALS AND METHODS
6.2.1 Chemicals and reagents
p-Nitrophenyl palmitate, sodium dodecyl sulphate (SDS) and gel filtration markers, coomassie brilliant blue R-250 bromophenol blue, phenylmethyl-sulfonylfluoride (PMSF), DEAE sepharose and sephacryl S-200 were obtained from Sigma Chemical Company, USA. All other chemicals used were of analytical grade and of the highest purity available locally.

6.2.2 Microorganism and lipase production
The bacterial strain, Staphylococcus arlettae JPRW-1 used in this study was isolated from a rock salt mine Darang HP, India and identified by 16S rRNA analysis.
*Staphylococcus arlettae* was cultured in LB medium (10 g of casein enzymic hydrolysate, 5 g of yeast extract, 10 g sodium chloride per litre) (pH 7). Submerged fermentation was carried out by seeding the spore suspension (50 ml) in Erlenmeyer flasks (1000 ml) containing 500 ml of the LB medium supplemented with soyabean oil (12 % v/v). The flasks were incubated at 35 °C under agitation (100 rpm) for 3 h. After incubation the fermentation medium was harvested by centrifugation at 6314 g for 10 min at 4 °C (Chauhan and Garlapati, 2013). The supernatant was collected and subjected to estimate the lipase activity.

### 6.2.3 Lipase assay and protein estimation

Lipase activity was assayed quantitatively using *p*-nitrophenyl palmitate as the substrate according to the method described by Garlapati and Banerjee, 2010. One enzyme unit was defined as the amount of enzyme that liberated 1 μmol of 4-nitrophenol per minute under the assay conditions. Protein content of cell-free supernatant was determined according to modified Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard.

### 6.2.4 Purification of *S. arlettae* lipase

#### 6.2.4.1 Ammonium sulphate precipitation

The cell-free culture supernatant was precipitated using solid ammonium sulphate to 60 % saturation (Chauhan and Garlapati, 2013). The pellet obtained after centrifugation (30 min at 8000 rpm) was dissolved in 10 ml of buffer (50 mM Tris HCl pH 8). Insoluble material was removed by centrifugation at 8000 rpm during 5 min. This is subjected to dialysis against 50 mM Tris–HCl buffer, pH 8 overnight.

#### 6.2.4.2 Ion exchange chromatography

The dialyzed material was directly applied on a DEAE-Sepharose column (15 × 1.6 cm i.d.), previously equilibrated with 50 mM Tris–HCl buffer, pH 8. The exchanged material was eluted with a linear gradient of NaCl ranging from 0.0 to 0.5M prepared in the same buffer at a flow rate of 60 ml/h and collected in 3 ml fractions. Fractions coming under the peak were pooled and dialyzed and their activity was studied. The
fractions with high specific activity were then pooled and concentrated for further purification.

6.2.4.3 Gel Filtration chromatography

The concentrated enzyme was placed on Sephacryl S-200 column (93cm×1.6cm i.d) that had been equilibrated with 50mM Tris–HCl buffer, pH 8 and developed at a flow rate of 30 ml/h. Finally, 3ml fractions were collected screened for enzyme activity. Active fractions were pooled for subsequent analysis.

6.2.4.4 Molecular Weight Determination through SDS-PAGE

The molecular mass of the denatured protein was investigated using SDS-PAGE (Amresco, USA). A SDS-12.5 % polyacrylamide gel was prepared according to the method of Laemmlili, 1970 with modification. Reference proteins for molecular mass determination (Biorad 161-0317) were lysozyme (14.4 kDa), trypsin inhibitor (21.5 kDa), carbonic anhydrase 31 kDa), ovalbumin (45 kDa), bovine serum albumin (66.2 kDa), phosphorylase b (97.4 kDa) b-galactosidase (116 kDa) and myosin 200 kDa). Protein bands were visualized by silver staining.

6.2.4.5 Peptide mass fingerprinting by MALDI-TOF-MS

Mix sample and matrix solutions 1: 2 in the tube and spotted on the sample stage Load 0.5-1.0 μl of premixed matrix, allow the mixture to air dry until all solvent is evaporated, usually less than 5 minutes. It was dried at room temperature then washed with 0.1 % TFA and was analyzed by Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Mass spectrometer was calibrated by peptide calibration standard II (Bruker). Acquired mass spectra had a resolution of ~6000 (FWHM), which was sufficient to identify the digested peptide. The mass/charge spectra obtained were searched in MASCOT search engine (http://www.matrixscience.com) using all the 3 databases (MSDB, SwissProt, NCBIInr). For search, peptides were assumed monoisotopic, oxidized at methionine residues and carbamidomethylated at cysteine residues, and peptide mass tolerance of 1.2 kDa was used for peptide mass fingerprinting.
6.3 RESULTS AND DISCUSSION

Enzyme purification is a series of processes intended to isolate a single type of enzyme from a complex mixture. Wide range of techniques are available and the choice depends on factors such as nature of the source i.e., extracellular or intracellular, scale of operation, stability of the enzyme etc. Success of enzyme purification scheme depends on the selection and effective combination of number of procedures. The objectives of such schemes are: high degree of purity, overall high recovery of enzyme activity and reproducibility of the process.

6.3.1 Purification and Molecular weight determination of lipase

The enzyme extract from S. arlettae has been subjected to a three-step purification scheme involving ammonium sulphate precipitation, ion exchange (DEAE sepharose) chromatography and gel filtration (Sephadex S-200). The enzyme extract was precipitated by ammonium sulphate for concentration. The precipitate was dissolved in minimum volume of tris buffer (pH 8.0) and dialysis was done in order to remove any salt concentration. Then it was loaded in the pre equilibrated DEAE - Sepharose column. Two major proteins peaks (A_{280}) was obtained in which most of the lipase activity was recovered in Peak 2 Fig. 6.1.

![Figure 6.1: Elution profile of lipase from DEAE-sepharose column.](image-url)
Lipase active fractions were pooled and concentrated. Gel filtration chromatography was followed after the ion exchange chromatography that facilitated salt removal and major impurities came while eluting the protein from the anion-exchanger column in the previous step. The protein was eluted as a single peak, the fractions were collected and concentrated (Fig 6.2).

![Figure 6.2: Elution profile of lipase from Sephacryl S-200 column](image)

Lipase activity and protein concentration were measured. Finally, a homogeneous lipase with specific activity of 32.5 U/mg and 27-fold of purification with 11 % yield was obtained. Purification results are summarized in Table 6.1.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Units</th>
<th>Total protein (mg)</th>
<th>Specific Activity(U/mg)</th>
<th>Purification fold</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>354.00</td>
<td>299.48</td>
<td>1.18</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitation</td>
<td>110</td>
<td>25</td>
<td>4.40</td>
<td>3.72</td>
<td>31.07</td>
</tr>
<tr>
<td>DEAE sepharose</td>
<td>88</td>
<td>5.2</td>
<td>17.6</td>
<td>14</td>
<td>24</td>
</tr>
<tr>
<td>Sephacryl S200</td>
<td>39</td>
<td>1.2</td>
<td>32.5</td>
<td>27</td>
<td>11</td>
</tr>
</tbody>
</table>

The eluent from the Sephacryl S-200 column yielded a single activity peak, and the protein was electrophoretically homogenous with a single band on SDS-PAGE. The purified lipase gave a single band of protein on 10 % SDS-PAGE (Fig. 6.3), indicating its homogeneity. A molecular weight of 45 kDa approximately has been estimated for
Staphylococcus arlettae. The molecular weight of S. arelette lipase was similar to that obtained from Mucor hiemalis (45 kDa) (Hio et al., 1999) and Staphylococcus aureus lipase was estimated to be 45 kDa by SDS-PAGE (Xie et al., 2012).

![Figure 6.3: SDS-PAGE analysis on the purified enzyme. Lane 1: molecular weight markers; Lane 2: crude enzyme; Lane 3: Partially purified lipase (after DEAE-Sepharose column chromatography); Lane 4: Purified lipase (after Sephacryl S-200 column chromatography).](image)

6.3.2 Peptide mass fingerprinting by MALDI-TOF-MS

Peptide-mass fingerprinting was carried out and data searched against NCBI databases showed that the peptide identified an amino acid sequence which shared similarity with α/β hydrolase fold gi|427702968 Cyanobium gracile PCC 630 covering 39 % of the entire sequence (219 amino acid residues). The MALDI-TOF peptide mass fingerprint (PMF) spectrum of fragments of lipase derived through trypsin digestion (in-solution). The sequence coverage of these fragments is shown in red, in the inset (Fig. 6.4). BLASTp analysis revealed similarities to other proteins Esterase/lipase Prochlorococcus marinus str. MIT 9303, Esterase/lipase Prochlorococcus marinus str. MIT 9313, Esterase/lipase Synechococcus sp. KS9916 provided 63 %, 64 % and 58 % identity respectively.
Conserved domains present in the encoded protein were also analysed using the NCBI Conserved Domain Search, which revealed a α/β hydrolase domain family. Bacterial lipases have a characteristic α/β hydrolase fold (Nardini et al., 1999). Homology was detected with lipase, the classical α/β hydrolase domain was identified between amino acids 137 and 185 which contains the putative catalytic serine. To verify that α/β hydrolase domain was indeed a serine hydrolase, a sample of enzyme was treated with the inhibitor PMSF which binds covalently to serine residues (Chauhan and Garlapati, 2013). Inhibition of activity with serine inhibitors shows that this lipase belongs to the class of serine hydrolases (Yadav et al., 1998 and Gilbert et al., 1991), which clearly indicated that a serine was involved in the catalytic mechanism.

### 6.4 Conclusions

In this study, the *S. arlettae* lipase was purified for the first time. The lipase was purified about 27 fold with molecular weight has been found to be 45 kDa approximately. MALDI-TOF-MS identified an amino acid sequence which shared similarity with α/β hydrolase gi|427702968 *Cyanobium gracile* PCC 6307.