Chapter 3

ISOLATION, PURIFICATION AND CHARACTERIZATION OF OVIS LACTOPEROXIDASE
3.1 Introduction

Lactoperoxidase is a glycoprotein with antibacterial properties; found in several biological fluids such as milk, tears, saliva, etc. The LP catalyses the peroxidation of endogenous thiocyanate to the antimicrobial hypothiocyanite ion (Hamon and Klebanoff, 1973). As such LP serves as a component of biological defense system of mammals. The prosthetic group of LP is a portoheme IX residue that is deeply buried in a crevice of the protein molecule (Sievers, 1979).

This section reports purification steps of sheep lactoperoxidase (sLP), its assay, and spectral studies.

3.2 Source of the Experiment Material

Sheep colostrum was collected from Sheep Breeding Research Station, Sandhinallah, Tamil Nadu. The breeding of sheep is seasonal in the tropics, one season ends in October and the other in March. The gestation period is around five months. Colostrum was collected, refrigerated for transport and kept frozen until use.

3.3 Experimental Procedures

Chemicals and reagents

1. 0.05 M Tris buffer (SISCO Research Laboratories, Bombay).
2. 0.05 M HCl.
3. 0.05 M Tris HCl buffer, pH 8.
4. 0.025 M NaCl added, 0.05 M Tris HCl buffer, pH 8.
5. 1 M NaCl added, 0.05 M Tris HCl buffer, pH 8.
6. C. M. Sephadex C-50 (Sigma).
7. Sephadex G 100 (Sigma).

Defatting of sheep colostrum was done by centrifuging at 10000 rpm for about 20 min. in a refrigerated centrifuge (REMI). Defatted colostrum was diluted in 1:1 ratio with 0.025 M NaCl added, 0.05 M Tris HCl buffer, pH 8. The diluted colostrum was added to already swelled C.M. Sephadex C-50 cation exchanger resin. The solution was kept in a refrigerator and stirred throughout the night.

3.3.1 Activation of CM Sephadex C-50

Cation exchanger CM Sephadex C-50 3 g (Sigma) was swelled by using 200 ml 0.025 M NaCl added, 0.05 M Tris HCl buffer, pH 8, for 2hrs.

Ion-exchange chromatography was done after diluting the colostrum in equal amount of 0.05 M Tris HCl buffer with 0.025 M NaCl, pH 8.

3.3.2 Activation Procedure of Dialysis Bag

Reagents for activation

1. 2% (w/v) sodium hydrogen carbonate in distilled water
2. 1 mM Ethylene Diamine Tetra Acetic acid (EDTA)
The dialysis bag was boiled in the above solution for 20 min. The dialysis bag was rinsed in distilled water. The bag was taken out and boiled in 1 mM EDTA for 10 min. then washed in distilled water and in 0.05M Tris HCl buffer, pH 8.

3.3.3 Ion Exchange Chromatography

In liquid chromatography, proteins are separated based on differences in their charge. This technique make use of specially modified beads whose surfaces are covered by amino groups or carboxyl groups and thus carry either a positive ($\text{NH}_3^+$) or negative (−COOH) charge at neutral pH. The proteins in a mixture carry various net charges at given pH. When a solution of protein mixture flows through a column of negative charge beads, only proteins with a net positive charge (basic protein) adhere to the beads, neutral and acidic proteins flow unimpeded through the column. The basic proteins are then eluted selectively by passing a gradient of increasing concentrations of salt through the column.

At low salt concentrations, protein molecules and beads are attracted by their opposite charges. At higher salt concentrations, positive salt ions bind to the negative charged beads, displacing the positive charged proteins. In a gradient of increasing salt concentration, weakly charged proteins are eluted first and highly charged proteins are eluted last.

The resin with bound proteins and supernatant solution was loaded onto a column (column length 45 cm, diameter 2.2 cm) and
the resin was allowed to settle well in the column. The tap was opened to drain the supernatant solution up to the upper level of resin length. The different proteins were eluted with NaCl gradients from 0.05 M to 0.5 M. The elute was collected from the column at flow rate of 25 ml h⁻¹ in 5 ml fractions. The experiment was carried out at 4°C. These fractions were stored in -20°C deep freezer. In the next step, the enzyme assay was done for different fractions.

3.3.4 Enzyme Assay using ABTS (2,2'-Aminobis(3-ethylbenzthiazoline-6-sulphonic acid)

Reagents
1. 0.1 mM 2,2'-Azinobis(3-ethylbenzthiazoline-6-sulphinic acid) (Sigma)
2. 0.1 M phosphate buffer, pH 6
3. 0.1 M phosphate buffer, pH 7
4. 0.1% gelatin
5. 3.2 mM hydrogen peroxide

The assay was carried out following the procedure of Shindler and Bardsley (1975). 3 ml of 0.1 mM ABTS in 0.1 M phosphate buffer pH 6 was mixed with 0.1 ml sample in 0.1 M phosphate buffer pH 7 containing 0.1% gelatin to initialize spectrophotometer (UV 1601 UV-Visible, Shimadzu, Japan) 3 ml 0.1 mM ABTS in 0.1 M phosphate buffer pH 6 was mixed with 0.1 ml sample in 0.1 M phosphate buffer pH 7 and 0.1 ml 3.2 mM H₂O₂ solution.
The absorbance was measured at 412 wavelength (nm) as a function of time for 2-3 min. The rate of change of absorbance was constant for at least 2-3 min.

3.3.5 Definition of Enzyme Unit Activity

One unit of activity is defined as that amount of enzyme catalyzing the oxidation of 1 μmol of ABTS min⁻¹ at 293 K (Molar absorbance coefficient 32400 M⁻¹ cm⁻¹). 1 M ABTS substrate gives an absorbance of 32400 M⁻¹ cm⁻¹ in 1 cm cuvette. 1μmol of ABTS gives an absorbance of 0.032400 (32400 x 10⁻⁶) i.e. one unit of activity that means 0.032400 absorbance per minute is equivalent to one unit of enzyme activity.

3.3.6 Dialysis

Protein solution in 0.05M Tris HCl buffer, pH 8 was taken in an activated dialysis bag and dialysed against distilled water in three times (8 hrs duration each). The length of the bag depends on the quantity of protein required. The amount of distilled water used for dialysis is ten times greater than the amount of protein solution taken in the dialysis bag. The protein fractions with peroxidase activity were pooled and concentrated by polyethylene glycol (PEG)-20000 or by lyophilization.
3.3.7 Concentration by PEG-20000

The protein fraction with peroxidase activity was taken in an activated dialysis bag. The dialysis bag, with the protein solution was placed on a clean petridish and the solid pellets of PEG-20000 were spreaded over the dialysis bag in the petridish. After 3 to 4 hrs, the protein solution was replaced in sterilized glass bottles.

The protein solution concentrated by PEG-20000 was checked for purity by Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE).

3.3.8 Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis

Electrophoretic experiments were performed using discontinuous buffer system described by Laemmli (1977) for checking the purity of the protein.

3.3.8.1 Preparation of stock solutions

1. Acrylamide- bisacrylamide solution

   30% (w/v) Acrylamide and 0.8% (w/v) N,N-methylene bisacrylamide in distilled water.

   Filter and store at 4°C in dark bottle.

2. Sodium dodecyl sulphate (SDS) 10% (w/v) in distilled water

3. Ammonium per sulphate (APS) (1.5% w/v) in distilled water.
4. Stacking gel buffer

0.5M Tris HCl, pH6.8

Tris buffer (6%, w/v) in 1 M HCl

Filter and store at 4°C

5. Resolving gel buffer

3M Tris HCl, pH8.8

Tris buffer (36.3%, w/v) in 1 M HCl

Filter and store at 4°C

6. N, N, N', N'-Tetra Methyl Ethylene Diamine (TEMED)

7. Reservoir buffer

0.25 Tris, 1.92 M glycine 1% SDS, pH 8.3

Tris buffer (30.3% w/v), glycine 14.40% (w/v) in distilled water and 10 g SDS made to one litre with distilled water.

Recipe for gel preparation using the SDS discontinuous buffer system (10%) is given in the table.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Stock solution</th>
<th>Resolving gel (ml)</th>
<th>Stacking gel (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Acrylamide-bisacrylamide 30:0.80</td>
<td>10 ml</td>
<td>2.5</td>
</tr>
<tr>
<td>2.</td>
<td>Resolving gel buffer</td>
<td>3.75</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>Stacking gel buffer</td>
<td>-</td>
<td>5.0</td>
</tr>
<tr>
<td>4.</td>
<td>10% SDS</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>5.</td>
<td>1.5% Ammonium per sulphate</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>6.</td>
<td>Distilled water</td>
<td>14.45</td>
<td>11.3</td>
</tr>
<tr>
<td>7.</td>
<td>TEMED</td>
<td>0.015</td>
<td>0.015</td>
</tr>
</tbody>
</table>
The resolving gel and stacking gel solution was fully degassed before adding SDS and TEMED by keeping it in a desicator and using vacuum pump apparatus. After adding APS and TEMED, resolving gel buffer mixture was poured in to spaces between slab plates at its two third length for gel casting. After polymerization (10-30 min.) stacking gel was poured into the space between slab plates, on the top of resolving gel for gel casting and inserted the comb without the formation of air bubbles to form sample wells. The comb was removed without distorting the shapes of the well from the plates after polymerization. A usual comb is for 13 wells, 0.7cm wide with 0.25 cm. spaces between the teeth. The gel was mounted in electrophoresis chamber and chamber was filled with reservoir buffer.

After pre-running the apparatus for 45 min., the wells were loaded with treated protein samples.

3.3.8.2 Treatment of protein sample

The protein sample (20 μl containing about 0.0063 mg of protein) was treated with 80 μl of sample buffer and boiled in a water bath for 3 to 5 min.

3.3.8.3 Sample buffer preparation

Sample buffer is prepared in the following composition.

0.05 M Tris HCl buffer, pH 6.8, 10% sucrose, 5% Mercaptoethanol, 5% SDS, 0.5% Bromophenol blue in distilled water.
3.3.8.4 Staining of gel

The gel after 6-7 hrs. running, was stained in a staining solution of Coomassie blue R-250 (0.1%) dissolved in water: methanol: glacial acetic acid (5:5:2 ratio by volume).

Staining was done for 12 hrs.

3.3.8.5 Destaining solution

After staining is complete, the excess stain is removed from the gel by using destaining solution of distilled water, methanol and glacial acetic acid in 5:5:2 ratio by volume.

Destaining was done till the bands become very clear. The gel after proper destaining was kept in 10% glacial acetic acid.

3.3.9 Purification by Gel Filtration

Usually, the protein after ion exchange chromatography contained a few contaminants. Concentrated sample by PEG-20000 was further purified by gel filtration on Sephadex G-100 column. sLP got eluted at 0.1M NaCl concentration.

3.3.9.1 Gel filtration

In this procedure which is another form of chromatography, the solution containing the mixture of proteins is passed down a column containing very small porous beads of a highly hydrated
polymer. The smaller protein molecules can penetrate into the pores in the beads and thus are retarded in their flow, down the column, but large protein molecules cannot penetrate into the beads and pass down the column more rapidly. Proteins of intermediate size will pass down the column at intermediate rates, depending on the degree to which they can penetrate into the beads. Such a gel filtration column is also called a molecular sieve.

3.3.9.2 Activation of Sephadex G-100

Sephadex G-100 (Sigma) beads for gel filtration prepared by cross linking dextran with epichlorohydrin. Dry beads size 40-120 μm. 4 g of Sephadex G-100 fresh powder was boiled at 90°C for 5 hrs in 0.05M Tris HCl buffer, pH 8.

3.3.9.3 Sephadex G-100 Chromatography

The Sephadex G-100 after swelling in 0.05M Tris HCl buffer, pH 8, degassed, was loaded on to column (length 50cm and diameter 1.1 cm). The buffer above the resin was eluted up to the level of resin. Then tap of the column was closed. 2 ml of concentrated protein solution was loaded on the top of the resin and the tap was opened. The elutant buffer of composition is the same as that of the resin swelling buffer, was used for eluting the protein. 20 fractions of 5 ml each were collected in sterile glass bottles at a flow rate of 30 ml h⁻¹. The experiments were carried out at 4°C.
The fractions were screened for peroxidase activity as done earlier. Fractions with peroxidase were checked by SDS-PAGE. After SDS-PAGE experiment, the fractions with peroxidase activity and single band on SDS-PAGE were pooled and concentrated by PEG-20000. Other fractions with LP activity were pooled and again applied to Sephadex G-100 column as done earlier, to get LP purified. The fractions were screened for peroxidase activity. Fractions with peroxidase activity were checked by SDS-PAGE. Native PAGE was done for specific staining of peroxidase. SDS was avoided in the latter procedure. Single band fractions were pooled, lyophilized and stored at -20°C.

3.3.10 Native PAGE

Non-dissociation discontinuous polyacrylamide gel electrophoresis was done at 10% gel composition, the recipe of which is shown below.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Stock solution</th>
<th>Resolving gel (ml)</th>
<th>Stacking gel (ml) APS as catalyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acrylamide-bis-acrylamide</td>
<td>10 ml</td>
<td>2.5</td>
</tr>
<tr>
<td>2</td>
<td>Resolving gel buffer</td>
<td>3.75</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Stacking gel buffer</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>1.5% Ammonium per sulphate</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>Distilled water</td>
<td>12.5</td>
<td>11.3</td>
</tr>
<tr>
<td>6</td>
<td>TEMED</td>
<td>0.015</td>
<td>0.015</td>
</tr>
</tbody>
</table>
3.3.10.1 Preparation of sample buffer for Native PAGE

Sample buffer is prepared in the following composition of 0.05 M Tris HCl buffer, pH 6.8, 10% sucrose, and 0.5% Bromophenol blue in distilled water.

3.3.10.2 Specific staining of peroxidase

Stain the gel with the following staining solution,

Benzidine (2.08% w/v) in 3% H₂O₂ and Acetic acid and distilled water in 1:4 ratio.

Bright blue colour bands were developed. When bands were stained sufficiently, the reaction was arrested by immersing gel in a large volume of 0.67% NaOH or 7% acetic acid solution for 10 min.

3.3.11 Determination of Protein Concentration

The protein concentration was determined according to the method given by Lowry et al., (1951) using crystalline BSA as standard and by spectroscopic method (Warburg and Christian, 1941).

3.3.11.1 Lowry's estimation of protein concentration

Reagents

1. Alkaline copper reagent

The following three reagents A, B and C are mixed freshly in the proportion 100:1:1 (v:v:v) respectively to get solution I.
A - 2% (w/v) sodium carbonate in 0.1 N sodium hydroxide solution

B - 1% (w/v) copper sulphate in distilled water

C - 1% (w/v) sodium potassium tartarate in distilled water

2. 0.1 N sodium hydroxide

3. Folin - Ciocalteau reagent

   1:1 ratio with distilled water

4. Standard protein solution

   100 mg% bovine albumin in 0.1 N NaOH solution

5. Working standard

   Dilute 1 ml of standard protein solution in 10 ml 0.1 N NaOH solution.

Procedure

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>0.2</td>
</tr>
<tr>
<td>Alkaline copper reagent</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Folin-Ciocalteau reagent</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

After adding alkaline copper reagent, the solution was kept for 10 min. and Folin-Ciocalteau reagent was added. The solution was mixed thoroughly and was allowed to remain for 30 min. Absorbance was read at 675 nm using spectrophotometer (UV-1601 Shimadzu, Japan).
3.3.11.2 Spectrophotometric method to determine protein concentration

The absorbance of each protein containing sample was measured at 280 nm ($A_{280}$) and 260 nm ($A_{260}$) on spectrophotometer. The protein concentration was then determined using the following relationship.

$$\text{Protein (mg ml}^{-1}) = 1.45 A_{280} - 0.74 A_{260}$$

3.3.12 Conformational study of sLP around metal through Electronic Spectra

3.3.12.1 Determination of electronic spectra

When a molecule absorbs ultraviolet or visible light of frequency, $\nu$ or wavelength, $\lambda$, an electron undergoes a transition from a lower to higher energy level in the molecule. The energy difference ($\Delta E$) is given by the expression.

$$\Delta E = h \nu = \frac{hc}{\lambda} \text{ kJ}$$

where $h$ is Planck’s constant and $c$ the velocity of radiation. Multiplication by Avogadro’s number $N$ ($6.02 \times 10^{23}$ mol$^{-1}$) will express the energy absorbed per mole. By inserting the numerical values for $h$ ($6.63 \times 10^{-37}$ kJ), for $c$ ($3 \times 10^{10}$ cms$^{-1}$) and using conversion factor (4.184) to convert kJ into kcal, the expression becomes

$$\Delta E \text{ kcal mol}^{-1} = \frac{119.75 \times 10^3}{\lambda \text{nm} \times 4.184}$$

for the region 200-750 nm, therefore, the energy required for electron transitions in range of 600-160 is kJmol$^{-1}$ for the
ultraviolet region 200-400 nm, the energy is of the same order of magnitude as the bond energies of common covalent bonds.

Energies of these magnitudes are associated with the promotion of an electron from a non-bonding \((n)\) orbital or a \(\pi\) orbital, to an antibonding \(\pi\)-orbital \((\pi^*)\) or to an antibonding \(\sigma\) orbital \((\sigma^*)\). The most important transitions in organic compounds are:

(a) \(\pi-\pi^*\) transitions, are usually associated with the multiple bonds of carbon with carbon, nitrogen, oxygen, sulphur, etc., and they generally give rise to high intensity absorption.

(b) \(n-\pi^*\) transitions, are usually associated with groups such as carbonyl, thiocarbonyl, nitroso, etc. Generally, the intensity of absorption is very much lower than that arising from \(\pi-\pi^*\) transitions.

The qualitative analysis of the spectrum will help to draw conclusions on several properties of the complex such as magnitude of ligand-field splitting, steriochemistry of the complex, possible distortion of symmetry environment, bonding characteristics and ligand field strength.

### 3.3.12.2 Experimental procedure

Electronic spectrum was recorded on UV-VISIBLE SPECTROPHOTOMETER (UV-1601, Shimadzu, Japan) with a data processor. The absorbance was measured between 200-400 nm using 1 cm. path length cuvette and a protein concentration of 6\( \mu \)M in 0.05 M Tris HCl buffer, pH 8.
3.4 Results

3.4.1 Purification Data

Many attempts have been made to isolate the peroxidase from different sources (Morrison and Allen, 1963; Iwamoto and Matsumura, 1966; Slowey et al., 1968; Rahemtulla et al., 1986, 1988; Rajesh Kumar et al., 1995; Marcozzi, 1996; Kobayashi et al., 1999; Shine et al., 2000). The enzyme lactoperoxidase was purified from the colostrum of sheep by relatively simple procedures including ion-exchange chromatography on CM Sephadex C-50 (cation exchanger), dialysis and gel-filtration chromatography on Sephadex G-100. The different stages of purification are summarized in Table 3.1. These procedures resulted in 2164-fold purification. From the colostrum, the percentage of the enzyme recovery was 13.9 with specific activity 92.2 IU mg\(^{-1}\) protein.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Purification steps</th>
<th>Total protein (mg)</th>
<th>Total activity (mg(^{-1}))</th>
<th>Specific activity (IU mg(^{-1}))</th>
<th>Recovery (%)</th>
<th>Purification fold (Cumulative)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Defat colostrum</td>
<td>16220.60</td>
<td>691</td>
<td>0.0426</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Ion exchange</td>
<td>2058</td>
<td>632</td>
<td>0.307</td>
<td>91.5</td>
<td>7.20</td>
</tr>
<tr>
<td></td>
<td>chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Dialysis</td>
<td>720</td>
<td>395</td>
<td>0.548</td>
<td>57.2</td>
<td>12.8</td>
</tr>
<tr>
<td>4</td>
<td>Gel filtration I</td>
<td>2.11</td>
<td>124.9</td>
<td>59.2</td>
<td>18.1</td>
<td>1389.6</td>
</tr>
<tr>
<td>5</td>
<td>Gel filtration II</td>
<td>1.043</td>
<td>96.2</td>
<td>92.2</td>
<td>13.9</td>
<td>2164.3</td>
</tr>
</tbody>
</table>
3.4.2 Enzyme Assay

There are various procedures to measure enzyme concentration (Pruitt et al., 1990). The guaiacol assay, pyrogallol assay (Chance and Maehly, 1955), and ABTS assay (Shindler and Bardsley, 1975) are commonly employed assays for peroxidase activity. Studies of peroxidase activity at different stages of purification using ABTS reagents have indicated the presence of LP in sheep colostrum. At low enzyme concentrations, there will be a significant enzyme reduction in enzyme activity, in the fluid phase, because of adsorption of the enzyme to container surfaces. This loss is prevented by including gelatin in the assay buffers as described under section 3.3.4. The specific activity of sLP was measured to be 92.2 IU mg⁻¹ at 30°C (pH 8) protein (Table 3.1).

3.4.3 Determination of Molecular Weight

Molecular weight of sLP was determined by method of Laemmli (1977) with a separating acrylamide gel of 10% (w/v) and stacking gel of 2.5% (w/v) containing 10% SDS. The enzyme was run in SDS-PAGE in the presence of molecular weight markers, and the result obtained has been shown as Figure 3.1. The standard marker proteins used in the experiment were phosphorylase b (94 KDa) human albumin (68 KDa) and ovalbumin (43 KDa). The single band was shown in the SDS-PAGE, which corresponds to a molecular weight of 73 KDa (Figure 3.1). Figure 3.2 shows the graphical representation to determine the molecular weight of sLP.
Figure 3.1 Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) of purified sheep lactoperoxidase. Purified sample and Molecular weight markers were applied on the top of the Gel. Lane (a) Molecular weight markers (Pharmacia) Phosphorylase b (94 KDa), Human albumin (68 KDa), Ovalbumin (43 KDa). Lane (b) Lactoperoxidase purified from colostrum of sheep (73 KDa).
Figure 3.2. Graphical representation of molecular weight determination of sLP

M1 – Phosphorylase b, M2 – Human albumin and M3 – Ovalbumin

3.4.4 UV-Visible Spectral Studies

Figure 3.3. UV-Visible spectrum of sLP
The UV-Visible spectrum of sLP gives absorption maxima at 280 nm and 412 nm (Figure 3.3). The absorption peak at 280 nm corresponds to $\pi \rightarrow \pi^*$ transition characteristic of aromatic amino acids. The absorption peak at 412 nm attributable to $^6A_{1g} \rightarrow ^4A_{1g}$ transition is characteristic of high spin octahedral Fe$^{3+}$ compounds (Lever, 1984).

### 3.5 Discussion

The purification scheme of sLP was ion-exchange chromatography on CM Sephadex C-50 (cation exchanger) and gel filtration chromatography on Sephadex G-100, a modification of earlier procedures done by Rajesh Kumar (1995). The sheep colostrum contains LP, which represents about 0.045% of total protein in the crude colostrum. An apparent 1390 fold of purification was obtained by gel filtration-I with 18% recovery. In the second step of molecular sieve chromatography an apparent 2164 fold of purification with 14% recovery was obtained. In the earlier report of LP from human colostrum, by chromatography on an immunoaffinity column, an apparent 1450 fold of purification was obtained in the single step with 21% recovery (Langbakkk and Flatmark, 1989). Gel filtration chromatography on the same column and under identical conditions resulted in sufficient purity (2164 fold of purification) for further analysis.

Sheep LP is obtained at higher degree of purity in two chromatographic steps. Molecular weight of LP from various sources such as Bovine LP, molecular weight 78 KDa (Sievers,
1980), Human SP molecular weight 75 KDa (Rahemtulla et al., 1988), Buffalo LP, molecular weight 73 KDa (Rajeshkumar et al., 1995) are found. Purity and molecular weight of sLP was determined by the method of Laemmli (1977) on SDS-PAGE and it corresponded to 73 KDa (Figure 3.1).

The procedure of Shindler and Bardsley (1975) was used for enzyme assay. Enzyme assay carried out at different steps of purification using ABTS has indicated the presence of LP in sheep colostrum. At low enzyme concentration, there is a reduction in enzyme activity due to the adsorption of enzyme to the container surfaces. This loss is prevented by including 0.1% gelatin in the assay buffer. The specific activity of sLP was measured to be 92.2 IU mg\(^{-1}\) protein at 30°C (pH 8).

The absorbance spectra of hog mucosa intestinal peroxidase and bovine LP resembled each other in every state of compound (Kimura and Yamazaki, 1979). Hog mucosa intestinal peroxidase resembles bovine LP in the spectral properties. The spectra of bovine LP were consistent with those reported by Theorell and Akeson (1943), Polis and Shmukler (1953), and Morrison et al. (1957). The similarities in the protein composition with regards to aromatic amino acids were confirmed by UV-Visible spectrum of SP and LP, which were identical with well defined peaks at 280 nm. The SP had the characteristic peroxidase heme spectrum in the range of 405-420 nm. The ratio between the absorbance of soret band (412 nm) and the absorbance at 280 nm was 0.81 (Rahemtulla et al., 1988).
In the spectrum of sLP as studied by UV-Visible spectroscopy, the well defined peaks at 280 nm indicated the similarities in the protein composition with regard to aromatic amino acids in bovine LP and SP. The sLP had the characteristic peroxidase heme peak at 412 nm identical with peroxidase heme peak of SP and bovine LP (Rahemtulla et al., 1988).