Chapter IV

Proteomic analysis of female goat urine during estrous cycle

4.1. Introduction

Urine is considered as a predominant source of communication in animals. Urine has been reported to contain various biochemical moieties, which encodes the message and favors the communication. The biochemical molecules consist of volatiles, proteins, lipids, fatty acids and other regulatory elements (Bouatra et al., 2013). Urine has been considered as a non-invasive biomarker in disease identification for centuries, since collection of urine never brings any stress to the animals. In biochemistry, the term urinalysis refers to the analysis of urine in terms of biochemical in turn compared with the standard values and makes them use to validate urine as a biomarker (Delanghe and Speeckaert, 2014). Thus, the process of excretion certainly not be an elimination of digested waste materials, but also provide insightful meaning in terms of advertisement of physiological status (Achiraman et al., 2011b). Each constituents present in the urine denote specific changes inside the body. In animals, the constituents possess additional roles; most important is the delivery of message to the conspecifics. Thus, the modulatory effect of these biochemical constituents paid greater interest in animal communication. For instance, volatile compounds present in the animal’s urine having the capacity to maintain social regulation, advertisement of dominance, mother-young interaction, and sexual communication and so on (Tirindelli et al., 2009).

Next to volatiles, proteins are the premier molecules studied extensively in small mammals. Proteins perform variety of functions inside the body, and a trace amount has been excreted in the urine (Berger and Szoka, 1981).
However, the presence of protein in higher concentration in urine signifies the critical condition called proteinurea. The fact was resolved and the breakthrough was discovered in animals that animals having permanent proteinurea not as an indicator of disease, but as a factor regulating the communication among conspecifics (Cavaggioni and Mucignat-Caretta, 2000). Initially, it has been reported that urine of mice and rat contains more amount of proteins and later it was discovered that the protein act as carrier for the volatile compounds. Later on the concept was well proved in both the rodents and follow-up structural and functional elucidation clearly determined the role of carrier protein in animal communication. It has been thoroughly reviewed by Flower, (1996) and (2000). Proteins excreted in the urine identified as a promising approach, its endocrine dependency further kindle its importance to study of urinary proteome and it was found that the male and female at estrus have higher concentration of proteins than that of other phases of female. It was proved that testosterone plays a major role in the regulation of excretion of particular protein, the so-called pheromone carrier protein (Ponmanickam et al., 2010). Similarly, estrogen also regulates the protein excretion in urine, since profound changes in the expression pattern of pheromone carrier protein was observed during different phases of estrous cycle. Thus, the phase change in the female’s reproductive cycle often associated with the regulation of excretion (Stopka et al., 2007). However, there are only limited reports available on the concentration of all the proteins rather than a specific protein.

The study of other proteins will provide additional information regarding the molecular mechanism and how the protein gets into the urine and for the purpose what it stands for. Also, the protein profile of urine may change during the different reproductive phases. Also, as like that of smaller mammals, the existence of particular proteins is not always possible in higher
mammals. Moreover, reports are available in the study of urinary proteome which is scarce, especially in goats. Hence, we here aimed to study the total proteome of female goat urine during four different phases of estrous cycle to bring out a better marker for differentiation of phases in addition to provide estrus-specific marker protein.

\section{Material and Methods}

\subsection{Estrus synchronization and urine collection}

The method of estrus synchronization and urine collection was followed as mentioned in chapter I. The urine samples were stored in ice condition and transported to lab and kept at \(-20^\circ\) C until further analysis.

\subsection{Protein analysis}

The urine samples were thawed to room temperature and were processed using molecular weight cut-off membrane (Vivaspin 20, 10 kDa MWCO, GE Healthcare). Twenty milliliter of each urine samples was poured into upper part of the individual tubes containing the cut-off membrane. The tubes were then centrifuged at 4000 rpm for 10 minutes (Eppendorf 450R). The centrifuge was repeated with similar time and rpm until minimum volume of sample in the upper layer. After centrifugation, the flow through was discarded and the samples retained in the upper part were taken for further analysis.

\textbf{Protein Estimation}

The total protein in the urine samples was determined by adopting the method of Bradford (1976) with Bovine Serum Albumin (BSA) as standard. To the required volume of protein sample, distilled water was added to make up the volume to 100 µl of Bradford reagent (100 mg of Comassie Brilliant Blue G 250 to 50 ml of 95% ethanol and 100 ml of 85% orthophosphoric acid, made up to 1000 ml) was added-extract and mixing thoroughly and allowed to
stand for 5 minutes. The absorbance was read at 595 nm in a Spectrophotometer.

**Composition of Bradford Reagent**

- Coomassie Brilliant Blue G-250
- Ethanol
- Acetic Acid

**Methodology**

Two microlitre of protein sample was added with 2.5 ml of Bradford reagent and incubated in dark for 10 minutes. After the appearance of clear blue colour, the readings were taken at 595nm in UV-Spectrophotometer.

**4.2.3. SDS-PAGE**

The protein samples processed using the cut-off membrane were used for SDS-PAGE analysis for protein separation. SDS-PAGE was prepared (12%) and each sample (50 µg) was loaded with the gel loading dye with the molecular weight marker. The molecular weight of the protein was compared with the molecular weight marker, and the protein of interest was cut out from the gel using sterile blade and was used for further analysis.

**Principle**

A special form of poly acrylamide electrophoresis is sodium dodecyl sulphate –poly acrylamide gel electrophoresis. In this technique, protein mixture is first denatured with SDS and β- mercapto ethanol, which results in reduction of the s-s-bridge in the protein and dissociation of the polypeptide chains. The SDS forms a complex with the polypeptides. These complexes have a strong negative charge that completely overshadows the charge of the polypeptide itself. The SDS polypeptide complexes carry an almost uniform charge density and also the form of the complexes is rather regular. Therefore,
the migration velocity through the gel is determined by the molecular mass of the polypeptide.

**Procedure**

The 12% SDS-PAGE was modified. The Polyacrylamide gels were cast between 11.5x10.3 cm glass plates. Liver, kidney and serum proteins were identified by running molecular mass reference standard (Bangalore Genei cat no: PMWM) containing phosphorylase- 97.7 kDa; serum albumin-66.0 kDa; ovalbumin-43.0 kDa; carbonic anhydrase-29.0 kDa; trypsin inhibitor-21.1 kDa; lysosome- 14.4 kDa respectively. Electrophoresis was carried out for 4 hours at room temperature.

**Sample preparation**

Prior to loading the SDS-PAGE, tissue extract was mixed with the 1x sample buffer with equal volume (1:1) and mixed with vortex mixer (CM 101, REMI equipment) for few seconds and kept for water bath at 100°C for 4 min, for the purpose to breakdown the polypeptides.

**Reagents required**

**Acrylamide solution (30%)**

- Acrylamide -14.6 g
- Bisacrylamide -0.4g
  
  Water make up to 50 ml (store at 4°C in dark room)

**Running gel buffer (lower Tris) : pH-8.8**

- 1.5 M Tris-Cl (MW-121.14) -9.075g
- Adjust pH with HCl
- Made up to 50 ml with distilled water
Stacking gel buffer (upper Tris): (pH-6.8)

1M Tris- Cl -3.029 g
Adjust pH with HCl
Made up to 50 ml with Distilled water

10% SDS

SDS -2.5 g
Made up to 25 ml with Distilled water

10% Ammonium per sulphate (APS)

APS -0.5 g
Made up to 5 ml distilled water

Tank buffer (1x): pH –8.2

Tris -750 mg
Glycine - 3.6 g
SDS - 250 mg
Made up to 250 ml with Distilled water

Sample buffer (1x)

Tris -1.25 ml of stacking gel buffer (pH-6.8)
SDS -2.0 ml solution of 10% SDS
Glycerol -1 ml
β-mercapto ethanol -0.5 ml
A pinch of bromophenol blue added as indicator dye. Water make up to 5 ml.

Staining solution: (Coomassie blue R-250) (0.5% stain)

45% Methanol -50 ml
10% Acetic acid -10 ml
Coomassie BlueR-250 -500 mg
Made up to 100 ml with Distilled water
De-staining solution

50% Methanol   -50 ml
10% Acetic acid -10 ml

Made up to 100ml with Distilled water

The gel was prepared by mixing the stock solution mentioned above.

Separating gel (12%)

Dis. Water                    -3.3 ml
30% Acrylamide                -4.0 ml
Tris (pH- 8.8)                -2.5 ml
10% SDS                       -0.1 ml
10% APS                       -0.1 ml
TEMED                         -0.002 ml

Stacking gel (5%)

Dis. Water                    -2.1 ml
30% Acrylamide                -0.5 ml
Tris (pH-6.8)                 -0.38 ml
10% APS                       -0.03 ml
10% SDS                       -0.03 ml
TEMED                         -0.003 ml

Preparation of gel

Two glass plates were sandwiched using 1.5 mm space strip and the bottom was sealed with 1% agar. The separating gel (12%) mixture was poured between the two glass plates and was allowed for polymerization. A few drops of separating gel- overlying solution were layered over the separating gel mixture. The solution was then decanted. Then the stacking gel was poured on the separating gel and the comb was inserted. After polymerization of stacking gel, the comb was removed and the wells were
rinsed with water. After removing the comb, the glass plates were fixed to the gel chambers.

**Solubilization of sample**

Samples were mixed with equal volume of sample buffer treated at 100° C for 4 minutes. Then, the samples were loaded into the wells of stacking gel. The chambers were filled with tank buffer and electrophoresis started. Initially a current of 60v was applied till the dye enters the separating gel subsequently current was increased to 100v. Electrophoresis was continued till the marker reached 1cm above the edge of separating gel.

**Protein detection by Coomassie Brilliant Blue stain**

Once the gel was completed it was rinsed with distilled water for 2 minutes and stained with 0.5% CBB-R-250 stain for 2 hours at room temperature. The stained gel was destained until the appropriate background was obtained. The gel was washed with distilled water and stored in the refrigerator for image analysis.

**4.2.4. In-gel Trypsin digestion protocol and MALDI spotting**

**Solutions needed**

- 100% Acetonitrile
- 100 mM ammonium bicarbonate

**Wash Solution**

- 50% acetonitrile and 50 mM ammonium bicarbonate

**Reduction solution**

- 10 mM DTT in 100 mM ammonium bicarbonate

**Alkylation solution**

- 50 mM iodoacetamide in 100 mM ammonium bicarbonate
**Trypsin solution**

20 μg/ml

**Extraction solution**

0.1% TFA and 50% Acetonitrile

The desired protein bands (stained gel pieces) were excised mince into pieces (1 -3 mm) and transferred into a sterile microcentrifuge tube. It was washed with 500 μL of wash solution (50% acetonitrile, 50 mM ammonium bicarbonate) and incubated at room temperature for 15 minutes with gentle agitation (vortex mixer on lowest setting). The solution is then removed with a pipette. The gel pieces were washed two more times with 500 μL of wash solution (15 minutes each) or until the Coomassie dye has been completely removed. The gel pieces were dehydrated in 100% acetonitrile for 5 minutes. When dehydrated, the gel pieces will have an opaque white color and will be significantly smaller in size.

Acetonitrile was removed with a pipette and then the gel was completely dried at room temperature for 10-20 minutes in a centrifugal evaporator. The gel pieces were rehydrated in 150 μL reduction solution (10 mM DTT, 100 mM ammonium bicarbonate) for 30 minutes at 56° C. The reduction solution was discarded with a pipette and 100 μL alkylation solution (50 mM iodoacetamide, 100 mM ammonium bicarbonate) was added and incubated for 30 minutes in the dark at room temperature. The alkylation solution was discarded with a pipette and 500 μL of wash solution was added and incubated at room temperature for 15 minutes with gentle agitation. The wash solution was discarded and dehydrated in 100 μL 100% acetonitrile for 5 minutes. Acetonitrile was discarded and the gel was completely dried at room temperature in a centrifugal evaporator. Typically, this is modified sequencing grade trypsin (Product number V5111, Promega, Madison, WI).
The lyophilized trypsin (20 µg/vial) was re-suspended in 1 mL of 50 mM ammonium bicarbonate, aliquot (50 µL/tube) and stored at -70° C.

The gel was rehydrated with a minimal volume of protease digestion solution. Approximately 20 µL solution was added for small gel plugs. The gel pieces were digested overnight at 37° C. The sample was centrifuged (12 kg for 30 sec). The supernatant was transferred (containing tryptic peptides) to sterile centrifuge tube. To that 25-50 µL of extraction solution (60% acetonitrile, 0.1% TFA) was added to gel pieces and sonicated in ultrasonic waterbath for 10 min. Alternatively, it was gently agitated by vortexing at lowest setting. The tubes were again centrifuged for 30 sec at 12 k g for 30 sec). The gels were extracted with an additional 25-50 µL of extraction solution. Then the gel pieces were agitated by sonicating in a waterbath for 10 minutes or with gentle vortexing.

Then the tubes were spin down and supernatant was transferred. The pooled extracted peptides were dried by centrifugal evaporation to near dryness. To this, 5 µL of re-suspension solution (50% acetonitrile, 0.1% TFA) was added and sonicated in water bath or gently agitated on a vortex at lowest setting. The samples were spin down and 0.5 µL was spotted on MALDI plate followed by 0.5 µL of alpha-cyano-4-hydroxycinnamic acid matrix (10 mg/mL in 50% acetonitrile, 0.1% TFA). The spots were allowed to dry completely and then the plates were loaded into Voyager. The plate was calibrated using internal tryptic peaks of 842.5 and 2211.1 Da.

**Note:** This protocol contains a reduction and alkylation step. Alternatively, this can be performed prior to gel electrophoresis or after first dimension isoelectric focusing. After peptide extraction mass spec analysis should be performed as soon as possible. Preparation of peptides must be performed with labware that has never been in contact with nonfat milk, BSA, or any
other protein blocking agent to prevent carryover contamination. Always use non-latex gloves when handling samples, keratin and latex proteins are potential sources of contamination. Never re-use any solutions, abundant proteins will partially leach out and contaminate subsequent samples.

**4.2.5. MALDI-TOF Mass Spectrometry**

The tryptic digests were prepared by mixing equal amounts (2:2) of peptide mixture with the matrix solution (α-cyano-4-hydroxycinnamic acid) saturated with 0.1% TFA and acetonitrile (1:1). Then the samples were analyzed in reflectron mode with delay time of 90 ns and 25Kv accelerating voltage in the positive ion mode. To improve the signal to noise ratio summation of 300 laser shots were taken for each spectrum. External calibration was done using peptide I calibration standard with masses ranging from 1046-3147 Da. Mass spectra were acquired using ULTRAFLEX-TOF/TOF mass spectrometer (Bruker Daltonics, Germany), equipped with a 337 nm pulsed nitrogen laser. MS-MS spectra were acquired by selecting the precursor mass with 8 Da window.

Spectra were processed using flex Analysis software. Monoisotopic peptide masses were assigned and used in the database search. The protein identification was accomplished utilizing the MASCOT data base search engine (Matrix Science, London, UK) (http://www.matrixscience.com, search engine). Probability-based MW search scores were estimated by a comparison of search results against an estimated random match population and were reported as 10 log10 (P), where P is the absolute probability. Scores >63 were shown to be significant (P<0.05) in the mascot search. Proteins identified with scores less than the significant level were reported as unidentified.
4.3. Results

4.3.1. SDS-PAGE

SDS-PAGE analysis of urine from estrous cycle revealed protein at different molecular weight with consistent variation in concentration. Proestrus urine revealed seven different proteins with the complete absence of 25 kDa protein and specific presence of 98 kDa, which is absent in other phases. In addition, the concentration of 68 kDa protein was higher in proestrus phase than that of other phases, which was markedly reduced in estrus phase. Estrus phase revealed the presence of seven different proteins with the specific presence of 25 kDa protein and absence of 98 kDa protein. The concentration of 25 kDa protein was further reduced to metestrus whereas it was completely absent during proestrus and diestrus. Metestrus phase also revealed protein profile as same as estrus. Diestrus phase revealed higher concentration of 68 kDa protein next to proestrus (Figure 4.1).

Fig. 4.1. SDS-PAGE (12%) analysis of urine samples from female goat during estrous cycle

L1- Diestrus, L2- Metestrus, L3-Estrus, L4- Proestrus, L5& L6- Marker.
4.3.2. MALDI-TOF analysis

The MALDI-TOF analysis of proteins and resulted peptide mass were analysed in MASCOT tool and the score value with sequence coverage were given in Table 4.1.

**MALDI TOF analysis of 25 kDa protein**

Estrus-specific protein of 25 kDa protein showed matching with Complement C3 (Fragment) with score value of 30 and sequence coverage about 6%. The sequences given below are the deduced peptide sequences obtained in MASCOT search. The deduced sequences were taken for BLAST search and the sequences matched with the existed protein are given in Figure 4.2 a and b.

```
LFPVTRQLNOP ILSSLVVVDIM NPDGVVDRI EKV MELRPFHVPA ITSLGDWK
AFTIHIIKAM HIYGKPVMGR LLL GQSLYVEASV ISSDAGEIED SILDDIPIVA
SPYSIKSK
```

**MALDI TOF analysis of 28 kDa protein**

The 28 kDa protein showed matching with Acetyl Co-A carboxylase with 7% sequence coverage and the score value of 52 (Fig.4.3 a & b). When the mass values were given as input in MASCOT tool the deduced sequences were obtained as given below. After that the sequences were taken BLAST search which showed the matching with Acetyl Co-A carboxylase protein.

```
MVMT LTAAESGCIH YVKRPGALDL PGCVIAKM DIMTSVSGRI
PPNVEKSIKK EMAQYASNIT SVLCQFPSQQ IANILDHAA TLNRSEREV
FFMNTQSIVQ LVQRYR GHMKAVVMDL LRQYLRVETQ FQNGHYDKCV
FALREENKSD MNTVLNYIFS HAQVTKK
```
MALDI TOF analysis of 32 kDa protein

The 32 kDa protein has showed score value of 54 with Transportin and the sequence coverage about 17% (Fig.4.4 a & b). The mass values resulted in the deduced peptide sequences as given below. When these sequence were taken for BLAST search, it showed matching with Transportin protein.

MEGAKP TLQL VYQAVQAL YH DPDP S GK VH AWEISDQL LQIRQD VESC
YFAAQTMKMK IQTSF YELPT DSHASLRDL SPVITQLAL AIADLALQMP
SWKGCVQTLV EK RTEII E DLAFYSSTV V SLLMTCVEK AGTDEK
VFRCLG SWFN LGVLDSNFMA NNK

MALDI TOF analysis of 42 kDa protein

The 42 kDa protein showed top score value of 63 with Unconventional Myosin VII-a and the sequence coverage of 33% (Fig.4.5 a & b). The deduced peptide sequence was obtained as mentioned below for the peptide mass of 42 kDa protein. The deduced sequences were taken for BLAST search where it shows 33% of sequence coverage with Unconventional Myosin VII-a.

YRDHLIYTYT G SILVAVNPY QLLSIYSPEH IRKIG EMPPHIFAL A
DNCYFNMKRN SRDGCCIISG ESGAGKT KLTLQFLAAI SGQHSWIEQQ
VLEATPILEA FGNKYID IHFNK KLGLGQATDY NYLAMGNCIT
CEGREDSQEY ANIRSA MK DRTF ENLDA CEVLF STALATAASL
LEVNP DLMN CLTSR
Table 4.1. Features of estrus-specific proteins identified using MASCOT tool

<table>
<thead>
<tr>
<th>S. No</th>
<th>Molecular weight of the Protein</th>
<th>Proteins matched by MASCOT</th>
<th>Score value in MASCOT search</th>
<th>Sequence coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25 kDa</td>
<td>Complement C3 (Fragment)</td>
<td>30</td>
<td>6%</td>
</tr>
<tr>
<td>2</td>
<td>28 kDa</td>
<td>Acetyl Co-A carboxylase</td>
<td>52</td>
<td>7%</td>
</tr>
<tr>
<td>3</td>
<td>32 kDa</td>
<td>Transportin</td>
<td>54</td>
<td>17%</td>
</tr>
<tr>
<td>4</td>
<td>42 kDa</td>
<td>Unconventional Myosin-VIIa</td>
<td>63</td>
<td>33%</td>
</tr>
<tr>
<td>5</td>
<td>55 kDa</td>
<td>Protein Wnt</td>
<td>47</td>
<td>56%</td>
</tr>
<tr>
<td>6</td>
<td>65 kDa</td>
<td>Interleukin</td>
<td>40</td>
<td>65%</td>
</tr>
<tr>
<td>7</td>
<td>74 kDa</td>
<td>Hemoglobin subunit epsilon</td>
<td>50</td>
<td>71%</td>
</tr>
</tbody>
</table>
a)

**Top Score:** 30 for **C01_RPTvH**, Complement C3 (Fragment) OS-Dptatetus burgeri OR-C3 PE-1 SW-1

**Mascot Score Histogram**

Protein score is \(-10 \times \log(P)\), where \(P\) is the probability that the observed match is a random event. Protein scores greater than 40 are significant (p<0.05).

![Mascot Score Histogram](image)

b)

VLVIAPAATS SYDDLAVAIL MVDQKKITEV HVLLVPNHTG ATLDEKKVKL QWDNKFIATF KLQVTPKEVE KWKEFDVRLM VKWDGGQHME IDIPLTSRRG LVFAQTDQPI YTPNNDVNIR **LFPVTRQLNOP ILSSLVVDIM NPDGVVVDR** EKNAFEVEKV **MELRPFHVPA ITSLGDW**IV SWMKDKPOFN YTSGFKVEEY VLPTFDVSIT SEQPYLHVYD **KAFTHIKAM HIYGKPV**MGR AYVRYGVKHQ SKRTLLSTSS ALARFEQGEA MHTLRQKHL EQYPDPKLLL **QGSLYVEASV ISSDAGEIED SILDDIPIVA SPYSIK**WT VPFFKPGYPY IYKVLVLPD

**Fig.4.2.**

a) Histogram of the Mascot score for 25 kDa protein showing the score value of 30 with Complement C3 (Fragment)

b) Number in the mass spectrum gives precise m/z (M+H) values for the detected peptide ion signals. The matched 20% sequence coverage is highlighted by being given in red color.
a)

Top Score: 52 for ACACA_SHEEP, Acetyl-CoA carboxylase 1 OS=Ovis aries GN=ACACA PE=2 SV=1

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 62 are significant (p<0.05).

b)

PSVLRSPSAG KLIQYIVEDG GHVFAGQCYA EIEVMKMVMT LTAAESGCIH YVKRPGAALD PGCVIAKMQL DNPSSVQQAE LHTGSLPRIQ STALRGEKLH RVFHYVLNDN VNMNGYCLP DPFFSSRVKD WVEGLMKTIR DPSLPLELQ DIMTSVGRI PPNVEKSIKK EMAQYASNIT SVLQFFPSEQQ IANILDHAA TLNRSEREF FFMNTOSSVQ LVQYRSGRGIR GHMKAVMML LHRQYLRTEDQ FQNGHYDkcV FALRENSKD MMTVLNYIFS HAOVTQKNNLL VIMLIDQLCG

**Fig. 4.3.**

a) Histogram of the Mascot score for 28 kDa protein showing the score value of 52 with Acetyl Co-A carboxylase 1.

b) Number in the mass spectrum gives precise m/z (M+H) values for the detected peptide ion signals. The matched 7% sequence coverage is highlighted by being given in red color.
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a)

Top Score : 54 for TWPO3_HUMAN, Transportin-3 OS=Homo sapiens GN=TWPO3 PE=1 SV=3

Mascot Score Histogram

Protein score is $-10^{\log(P)}$, where $P$ is the probability that the observed match is a random event. Protein scores greater than 62 are significant ($p<0.05$).

![Mascot Score Histogram](image)

b)

MEGAKPTLQL VYQAVQALYH DPDPSGKERASFWLGELQRS VHAWEISDQL
LQIRQDVESC YFAAQTMKMK IQTSFYELPT DSHASLRDSL LTHIQNLKDL
SPVIVTQLAL AIADLALQMP SWKGCVQTLV EKYSNDVTSL PFLLEILTVL
PEEVHSRSRL IGANRTEII EDLAFYSTTV VSLLMTCVEK AGTDEKMLMK
VFRCLGSWFN LGVLDNFMA NNKLLALLFE VLQDKTSSN LHEAASDCVC

Fig. 4.4.  a) Histogram of the Mascot score for 32 kDa protein showing the score value of 54 with Transportin.

b) Number in the mass spectrum gives precise $m/z$ (M+H) values for the detected peptide ion signals. The matched 17% sequence coverage is highlighted by being given in red color.
a) Histogram of the Mascot score for 42 kDa protein showing the score value of 63 with Unconventional myosin VII-a (Fragment).

b) Number in the mass spectrum gives precise m/z (M+H) values for the detected peptide ion signals. The matched 17% sequence coverage is highlighted by being given in red color.
MALDI TOF analysis of 55 kDa protein

The 55 kDa protein has showed score value of Protein Wnt and the score value of 56% (Fig.4.6 a & b). The peptide mass value of the 55 kDa protein was taken for MASCOT search which resulted in the deduced peptide sequence as mentioned below. The sequences subjected to BLAST search for identifying the matching sequences which showed the 56% sequence matching with Protein Wnt.


MALDI TOF analysis of 65 kDa protein

The 65 kDa protein has showed the score value of 40 with Interleukin and the sequence coverage of 65% (Fig.4.7 a & b). MASCOT search of peptide mass values of the 65 kDa protein showed the peptide sequences as mentioned below. The sequences were subjected to BLAST search to exploit the highly matching protein and it shows about 65% of sequence coverage with Interleukin protein.


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Mascot Score Histogram

Protein score is $-10^*\log(P)$, where $P$ is the probability that the observed match is a random event. Protein scores greater than 61 are significant ($p < 0.05$).

**Fig. 4.6.**

a) Histogram of the Mascot score for 55 kDa protein showing the score value of 47 with Protein Wnt-2.

b) Number in the mass spectrum gives precise m/z (M+H) values for the detected peptide ion signals. The matched 56% sequence coverage is highlighted by being given in red color.
MAKVPDLFED LKNCYSENED YSSEIDHLSL NQKSFYDASY EPLREDQMNK
FMSLDTSETS KTSKLSFKEN VVMVAASGKI LKKRRSLNQ FITDDDELAI
ANNTEEIIK PRSAHYSFQS NVKYNFMVRVI JQECILNDAL NQSIIRDMSG
PYLTATTLNN LEEAVKFDMV AYVSEEDSQL PVTLRISKQ LFVSAQNEDE
PVLLKEMPET PKIIKDETNL LFFWEKHGSM DYFKSVAHPK LFIATKQEKL
VHMASGPPSI TDFQILEK

Fig. 4.7. a) Histogram of the Mascot score for 65 kDa protein showing the score value of 40 with Interleukin-1 alpha.

b) Number in the mass spectrum gives precise m/z (M+H) values for the detected peptide ion signals. The matched 63% sequence coverage is highlighted by being given in red color.
**MALDI TOF analysis of 74 kDa protein**

The protein at 74 kDa has showed the score value with Hemoglobin subunit protein-epsilon with the sequence coverage of 71% (Fig.4.8 a & b). The peptide mass of the 74 kDa protein showed the deduced peptide sequences as mentioned below. When the deduced sequence was subjected to BLAST search to identify the sequence coverage it shows 71% with the Hemoglobin subunit protein-epsilon.

\[
\begin{align*}
TILSVWGKFFDNFGNLSSP\text{AIMG}NP\text{V} & \text{KVL}TSFCEAVKAFAKLS \\
\text{ELH}CDKLHVDPENFKLLGNA & \text{MVILATHFG KEETPDVQAA WQKLVSGVAT}
\end{align*}
\]

ALAHKYH
4.4. Discussion

Proteins are the second major macromolecules involved in various biological functions. They have crucial role in animals both by presence or absence. Liver is the major factory for the synthesis of many proteins, where it has been transported to various organs to perform its function. At some
instance, proteins are excreted purposely in the excretory products mainly, through urine. The condition has been termed as proteinurea, an unusual condition denoting the status of illness or diseased condition. However, in animals, proteinurea has been observed permanently and later it was discovered that some of the proteins present in the urine have notable function, particularly in communication. The proteins perform the role of communication are said to be the carrier proteins, often have low molecular weight. Its structure and biological functions have been reviewed thoroughly by Flower (1996) and (2000). In smaller mammals, such as mice and rats exhibit a protein at the molecular weight ranging from 18-22 kDa have been proved as a carrier for the volatile molecules (Cavaggioni and Mucignat-Caretta, 2000). Later on it was discovered that hamster contain a vaginal secretory protein, elephant have albumin and human being possess apolipoprotein D as carriers for volatile molecules (Briand et al., 2004; Zeng et al., 1992). Since, the volatile molecules have significant biological functions, study of volatile-associated molecule, especially the carrier protein has opened a new window in the era of reproductive biology. Since, we have identified biochemical moieties and volatiles in the previous chapters of the thesis, it was suspected and that the urine may contain protein and that may have role as carriers for volatile molecules or its specific presence among different phases of the estrous cycle could consider as a better choice in the estrus detection.

We subjected the urine of female goats to analyse the protein profile. We observed difference in the expression profile of proteins among four different phases of estrous cycle. As we found protein concentration varied significantly among different phase of estrous cycle, the present data has additional clue that the physiological intervention play a major role and control the excretion of protein through urine. In support of this,
Muthukumar et al. (2013) identified proteins at four different phases of estrous cycle in mice, but they found concentration dependent of excretion of similar type of proteins. They further inculcate that a specific protein at 14.5 kDa have profound variation among different phases, and in particular estrus and metestrus phase contained higher concentration of that particular protein. In contrast, we found more proteins at higher concentration in proestrus. In addition, we found a protein at 25 kDa which was specific to estrus and metestrus phase and it was completely absent during proestrus and diestrus. However, diestrus phase revealed a good expression of 65 kDa protein next to proestrus. This is in accordance with the report of Ponmanickam et al. (2013) that the vaginal mucus protein concentration was higher in diestrus next to estrus. Excluding the 25 kDa protein, all the other proteins as same as in all the phases however the expression pattern was varied in every phases. For instance, 65 kDa protein highly expressed in proestrus which was remarkably reduced in estrus phase.

To further elucidate the specific role govern by each protein, we next sought to characterize the proteins estrus urine. We observed the estrus specific protein of 25 kDa with high score value with complement C3 protein. Uterine luminal fluid is responsible for transport and support medium for spermatozoa. It was studied that the secretion of luminal fluid is hormone-dependent, especially estradiol than progesterone, and hence it play a major role in implantation. It has also been proved that under the control of estrogen, the uterus has good quantity of luminal fluid. Florescin lebelled dextran was used to study the movement of the fluid, where under the surge of estradiol the concentration of fluid was higher, but dropped significantly when estradiol was reduced in presence of high level of progesterone (Selleh et al., 2005). It is interesting to note that the presence of complement C3 was observed in the uterus luminal fluid collected during estrous cycle of mouse.
The expression of complement C3 protein was proved to be under the control of estrogen, and hence, detectable amount of mRNA was observed during estrus and undetectable during metestrus and diestrus (Li et al., 2002). It is also substantiated with the ovariectomized mice with exogenous administration of estradiol. However, the level of complement C3 was again reached a detectable quantity during late pregnancy and lasts up to delivery (Li et al., 2002). In line with this, the 25 kDa protein of the present study showed high score value with Complement C3. It was interesting to note that the protein was appeared during estrus and metestrus, and, hence, it could be postulated that the 25 kDa protein has some crucial role during estrus and metestrus phase.

The second protein at estrus phase 28 kDa protein has showed top score with Acetyl Co-A carboxylase. In literature, it provides melonyl- Co A for the synthesis of fatty acids. Acetyl Co- A carboxylase was found in the mammary gland of goat and its expression was correlated with the production of milk (Travers and Barber, 1993). However, none of the reports were available in regard to the presence of this protein in urine. Since, this protein is presented during all the phases this may considered as female-specific protein which may confer important role in the synthesis of fatty acids in goats, however its presence and role in urine has to be studied in detail. The protein at 32 kDa has showed top score with Transportin. Eventhough there are only scanty reports are available regarding this protein, it has been reported that it play crucial role in neurodegenerative diseases namely, Amyotrophic lateral sclerosis and frontotemporal dementia (Brelstaff et al., 2011). The fourth protein at 42 kDa has shown top score value with Unconventional Myosin VII-a. This protein has several important roles in mammalian species and mutation of this gene revealed several impairments in mice (Gerdin, 2010). However, its presence in goats or urine has not been
reported. The fifth protein at 55 kDa showed top score with Protein Wnt, a
protein having signaling property. They pass the signals from outside of the
cell to inside through cell surface receptors. Wnt proteins have several notable
functions in physiology, including development of embryos and interplay in
several important diseases including cancer (Moon et al., 2004). This receptor
protein, otherwise termed as Frizzleds have many characteristics similar to G-
protein coupled receptor (Wang and Malbon, 2004). Thus, in our study we
speculate that Wnt protein may have notable function in intracellular
communication.

The protein at 65 kDa of the present study has top score value with
Interleukin, a group of proteins known to be involved signaling mechanism.
Its function has been implicated well in immune system (Staal et al., 2008).
However, no other specific functions have been studied in terms reproductive
biology for this protein, and hence, the presence of this protein in the urine of
goats has to be validated further and studied in detail to elucidate the
functional aspect of this protein in goats. The 74 kDa protein at estrus phase
showed top score with Hemoglobin subunit epsilon. Hemoglobin is expressed
in the embryo for the function of transport of oxygen. In addition, the embryo
is known to contain hemoglobin subunit epsilon. Taken together, the function
of the protein could be correlated with the oxygen transport and also it is
surprising to note that the protein was expressed in the female goats.

Even though MALDI-TOF and MASCOT analysis of estrus-specific
protein provide a fundamental data for identification of proteins, further study
at peptide level is warranted.
4.5. **Summary**

- The urine sample of female goats collected during estrous cycle were analysed for protein profile.
- The estrus urine sample revealed the presence of 25 kDa protein which was absent during pro-estrus and diestrus. However, the intensity of 65 kDa protein was higher in proestrus and reduced in estrus.
- Overall all the phases expressed similar pattern of proteins except fewer proteins.
- The proteins at estrus phase was analysed for peptide mass fingerprint by MALDI-TOF analysis revealed the 25 kDa protein as Complement component C3. The 32 kDa protein has showed score value with Transportin.
- The 42 kDa protein showed top score value with Unconventional Myosin VII-a and 55 kDa protein has showed score value with Protein Wnt.
- The 65 kDa protein has showed the score value with Interleukin and the sequence coverage of 65%. The protein at 74 kDa has showed the score value with Hemoglobin subunit protein-epsilon with the sequence coverage of 71%.