Expression profiling and biochemical characterization of rat lysozyme-like proteins.
INTRODUCTION

Proteins secreted by the epithelial surfaces of the male reproductive tract contribute to its physiological function. Examples include CRISP1, 2 and 4 (Ernesto et al. 2012), SAMP32 (Hao et al. 2002), cystatin C (Jiborn et al. 2004), SPAG11 family members (Yenugu et al. 2004) and CRES (Syntin & Cornwall 1999). Further, lysozymes are also one such group of proteins secreted by the male reproductive tract. Lysozymes are present in most of the biological fluids including semen (Mårdh & Colleen 1974). Measuring lysozyme levels in semen was recommended as a marker of infertility (Kuz’min et al. 1991). Origin of lysozyme present in the semen is very interesting. Analysis of the prostate and seminal vesicle fractions indicate that it is secreted equally by these two glands (Tauber et al. 1976).

Lysozymes are divided into six families based on their distribution and functions. They are g-type, i-type, c-type, plant, phage and bacterial. The c-type lysozyme is the predominant type and widely expressed in many species. Some of the species in the animal kingdom possess more than one type of lysozyme. For example mollusks possess both g-type and i-type lysozymes (Lien CalleWaert et al. 2010). The expression pattern of individual lysozymes seems to vary from species to species (Irwin & Gong 2003). Though the primary structure of these proteins are diverse, their three dimensional structure seems to be very similar (Wohlkönig et al. 2010). A common feature of most lysozymes is that they possess antibacterial activity. In spite of their strong binding ability towards lipopolysaccharide, they are more potent against Gram positive bacteria than Gram negative bacteria (Ohno & Morrison 1989). It is interesting to note that the decapeptide of lysozyme despite the absence of muramidase activity site, possess antibacterial activity. Few of the i-type lysozymes are bifunctional with both muramidase and isopeptidase activity. The isopeptidase activity contributes to the breakdown of the isopeptide bond formed between glutamine and lysine during blood clotting thereby a role in regulating blood flow.
Recently a group of lysozyme-like (Lyzl) genes that belong to c-type lysozyme family were identified. Zhang et al., have reported the mRNA expression of Lyzl2, Lyzl3, Lyzl4 and Lyzl6 in humans tissues. Lyzl2, Lyzl4 and Lyzl6 mRNA were found to be expressed only in the testes and Lyzl3 was found to be expressed in testes and pancreas (Zhang et al. 2005). Sperm lysozyme-like protein 1 (SLLP1 or LYZL3) was found to be an intra-acrosomal and non-bacteriolytic c-type lysozyme-like protein in human spermatozoa (Mandal et al. 2003). LYZL4 is a sperm bound protein with a role in fertilization (Sun et al. 2011) and is expressed in testes and epididymidis. These studies point to the fact that LYZL proteins are expressed predominantly in male reproductive tract. However in-depth analysis of their expression pattern and biochemical functions in general physiology and in the male reproductive function in particular are lacking. Hence, in this part of the study we attempted to characterize the expression of Lyzl genes and their protein products. Biochemical characterization was also undertaken to understand their role in general physiology.
MATERIALS AND METHODS

Total RNA isolation

Wistar rats aged 90 days were obtained from National Centre for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad. The animals were sacrificed and the following tissues were collected: brain, heart, lungs, liver, kidney, spleen, ovary, uterus, cervix, caput, corpus, cauda, testis, seminal vesicles and prostrate. The tissues were immediately snap frozen and stored at -80°C. Total RNA was extracted using TRIzol reagent (Invitrogen) (Chomczynski & Sacchi 2006). Briefly, 10 mg of tissue was ground with liquid nitrogen to make fine powder and 1 ml of TRIzol was added and vortexed for 10 min. The homogenate was centrifuged at 10,000 rpm for 10 min at 4°C. 200 µl of chloroform was added and mixed well and centrifuged for phase separation. The supernatant was then collected and equal quantity of isopropanol was added and centrifuged. The resulting pellet was then washed twice with 70% ethanol and air dried before dissolving in DEPC treated water. The purified RNA was then quantified using NanoDrop (Thermo Scientific) and the quality of the RNA was confirmed by agarose gel electrophoresis.

cDNA generation

To 2 µg of total RNA, 0.2 µg of oligo dT was added and the final volume was made upto to 10 µl with water. The mixture was incubated at 65°C for 5 min and a cocktail (containing 10 U reverse transcriptase (Promega), 10 U RNase inhibitor (Fermentas), 5X reverse transcriptase buffer, 10 mM dNTPs and water) was added and subjected to thermal cycling in a thermal cycler as per the manufacturer’s instruction. The cDNA generated was stored at -20°C.

Polymerase chain reaction

Gene specific primers for rat *Lyzl*, *Lyzl3* *Lyzl4* *Lyzl5* *Lyzl6* and *Lyzl7* were designed, synthesized (Table 7) and used for polymerase chain reaction (PCR) to study *Lyzl* gene
expression in various tissues. The number of cycles to amplify each cDNA in the linear range was determined by preliminary PCR under the following conditions: 94°C for 1 min followed by 30 cycles at 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec, and with a final round of extension at 72°C for 10 min. The internal control gene glyceraldehyde 3 phosphate dehydrogenase (Gapdh) was amplified with the same conditions. PCR amplified gene products were analysed by electrophoresis on 2% agarose gels.

Table 7: Gene specific primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5' ----&gt;3')</th>
<th>No. of bases</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyz1 FP</td>
<td>TGTCGGTGCTTCTGCCCCTAAT T</td>
<td>22</td>
<td>408</td>
</tr>
<tr>
<td>Lyz1 RP</td>
<td>GAC GAG TCT TTG CTC TCA CAG T</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Lyz3 FP</td>
<td>TCC AGC AAG GCC AAG GTC TTC A</td>
<td>22s</td>
<td>398</td>
</tr>
<tr>
<td>Lyz3 RP</td>
<td>TAG AAG TCA CAG CCA TCC ACC CA</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Lyz4 FP1*</td>
<td>ATG TGG GCA CTG TTG ACA CCA</td>
<td>21</td>
<td>602</td>
</tr>
<tr>
<td>Lyz4 RP1*</td>
<td>CTA CAC CAT TGA TCC TGC TCC A</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Lyz4 FP2*</td>
<td>GTG GTG ATT GAG GAT TCC TTC AG</td>
<td>21</td>
<td>641</td>
</tr>
<tr>
<td>Lyz4 RP2*</td>
<td>ATG GAG GCA CCA ATC GGA GTC A</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Lyz4 FP3*</td>
<td>ATG CAG CTG TAC CTG GTG CTT CT</td>
<td>23</td>
<td>603</td>
</tr>
<tr>
<td>Lyz4 RP3*</td>
<td>GCT GGT TATTTCTGCACCTTGTAC C</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Lyz5 FP</td>
<td>CACGCATGCAAGATTTAGTGAAGCTGAG</td>
<td>29</td>
<td>420</td>
</tr>
<tr>
<td>Lyz5 RP</td>
<td>CAGGTGACTCCACAGTCTCATCATAGT</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Lyz6 FP</td>
<td>TAT CTG TGT GGT GAG CTG CCT TCT</td>
<td>24</td>
<td>322</td>
</tr>
<tr>
<td>Lyz6 RP</td>
<td>TGC ACA GTG GAT GGA TGGAAT GAG</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Lyz7 FP</td>
<td>TATGCATGCTACAGAGTTTACAAAATGTGA</td>
<td>30</td>
<td>441</td>
</tr>
<tr>
<td>Lyz7 RP</td>
<td>AGGTCGACTTTAGGGAACAGGTTTCTGAAT</td>
<td>31</td>
<td></td>
</tr>
</tbody>
</table>

FP- forward primer; RP-reverse primer; *-used for amplifying the full length sequence.
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To study the androgen regulation of *Lyzl* transcripts, epididymides were obtained from sham operated, castrated and testosterone supplemented Wistar rats (n = 5 in each group). Testosterone supplementation was by a 20 mg dihydrotestosterone pellet implanted subcutaneously immediately after castration. The animals were sacrificed 14 days after castration. All procedures involving animals were performed in accordance with the guidelines established by the institute’s animal ethical committee (IAEC). For studies on the developmental regulation of *Lyzl* genes, testes and epididymides were obtained from 10- to 60-day old Wistar rats.

**Cloning of rat *Lyzl* genes**

The full length cDNA of rat *Lyzl* 1, 3, 4, 5, 6 and 7 without the signal peptide were amplified and cloned into pQE80m vector.

**Table 8: Cloning details**

<table>
<thead>
<tr>
<th>Gene</th>
<th><em>Lyzl</em> 1</th>
<th><em>Lyzl</em> 3</th>
<th><em>Lyzl</em> 4</th>
<th><em>Lyzl</em> 5</th>
<th><em>Lyzl</em> 6</th>
<th><em>Lyzl</em> 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insert size (bp)</td>
<td>687</td>
<td>684</td>
<td>674</td>
<td>717</td>
<td>687</td>
<td>720</td>
</tr>
<tr>
<td>Restriction sites used</td>
<td><em>Sph</em>1, <em>Sal</em>1</td>
<td><em>Sph</em>1, <em>Sal</em>1</td>
<td><em>BamHI, HindIII</em></td>
<td><em>Sph</em>1, <em>Sal</em>1</td>
<td><em>Sph</em>1, <em>Sal</em>1</td>
<td><em>Sph</em>1, <em>Sal</em>1</td>
</tr>
<tr>
<td>MW of the recombinant proteins (kDa)</td>
<td>16.2</td>
<td>17.27</td>
<td>15.4</td>
<td>17.7</td>
<td>16.7</td>
<td>17.27</td>
</tr>
<tr>
<td>pI of the recombinant proteins</td>
<td>8.59</td>
<td>6.28</td>
<td>7.02</td>
<td>6.17</td>
<td>6.43</td>
<td>5.58</td>
</tr>
</tbody>
</table>
Using gene specific primers, the coding region of Lyz1 genes without their signal peptide was amplified from testes cDNA. The amplified PCR products were purified and digested using appropriate restricting enzymes (Table 8). The digested PCR products were then purified, quantified and ligated into pQE80m vector. After ligation, each plasmid was transformed into E.coli DH5α and ampicillin resistant colonies were checked by colony PCR for presence of the insert. Plasmid was isolated from positive clones that showed right amplicon size. They were then sequenced to confirm the presence of the insert, its orientation and reading frame. Details of restriction sites used, size of the insert and the predicted size of the recombinant protein are given in table 8.

**Recombinant protein production**

Plasmids containing one of the Lyz1 coding region were transformed into E.coli BL21 to express the recombinant protein. The bacterial cells were then plated on Luria Bertani (LB) agar plates containing ampicillin as the selection marker. Single colony was inoculated into LB medium and incubated over night at 37°C for 16 h with shaking. 2% of the overnight culture was inoculated into 2 L of fresh LB medium. The culture was allowed to grow until it reaches an A_{600} = 0.5. Protein expression was induced with 1mM IPTG for 3 hr. After induction, bacterial cells were pelleted and stored at -80°C. The cytosolic and inclusion body fractions of the bacterial cells were checked to identify the localization of the recombinant protein. Since the LYZL proteins were present in the inclusion body fraction, they were purified under denaturation conditions. Briefly, 5 ml of buffer A (6M Guanidine HCl, 0.1M NaH₂PO₄, 0.01M Tris HCl, pH 8) was added to 1 g of bacterial cell pellet and lysed for 30 min, followed by centrifugation at 10,000 rpm for 30 min at 4°C. The supernatant was then incubated with 1 ml of 50% Ni-NTA resin which was prewashed with buffer A. After binding for 30 min, the column was packed and washed sequentially with urea buffers of gradient pH (buffer B (8M Urea, 0.1M NaH₂PO₄, 0.01M Tris HCl, pH 8),
buffer C (buffer B at pH 6.3) and buffer D (buffer B at pH 5.9)). The recombinant protein was then eluted with buffer E (buffer B at pH 4.5) after washing with buffer D1 (20mM Tris, 20mM Imidazole, 5mM BME, 500mM NaCl, 10% glycerol pH 5.8) to remove the non-specific proteins. Purity of the eluted fractions were checked by separating them on SDS-PAGE and visualizing the bands by coomassie brilliant blue staining.

The identity of the purified recombinant protein was confirmed by Western blotting using anti-His tag antibody. Protein fractions separated on 15% SDS-PAGE and electrotransfered to PVDF membrane at 25V for 16h were probed with rabbit anti-His antibody (Santa Cruz Biotechnology) and then incubated with anti-rabbit secondary antibody (Bangalore Genei) tagged with HRP. Protein bands were detected using chemiluminescence kit (GE healthcare). The fractions that contained the protein of interest were dialysed extensively at 4°C against 10 mM sodium phosphate buffer, pH 7.4.

**Raising of polyclonal antibodies**

New Zealand white rabbits aged four months were obtained from National Institute of Nutrition, Hyderabad and acclimatized to animal house conditions for 1 week. Preimmune serum was collected and checked for cross reactivity with recombinant LYZL proteins and male reproductive tract tissue lysates by Western blotting. After confirmation of non-cross reactivity, rabbits were immunized intradermally with 600 µg of the recombinant protein mixed with equal volume of Freund’s complete adjuvant. Booster doses containing 600 µg protein was mixed in Freund’s incomplete adjuvant were administered on 3rd and 28th day. Antibody titer was checked on 35th day and final bleeding was done on 42nd day (Xiao et al. 2004). Each antibody was checked for cross reactivity against each and every LYZL protein to ensure there is no cross reactivity within recombinant LYZL proteins.
Immunoblotting

Testes, caput, cauda, seminal vesicles and prostate tissues were collected from 90 day old Wistar rats and 10% homogenates were prepared in RIPA buffer containing protease inhibitors. The homogenate was then centrifuged at 10,000 rpm for 10 min to remove the debris and concentration of the protein in the supernatant was quantified by Lowry’s method. For sperm lysate preparation, 5 X 10^6 spermatozoa were suspended in 1 ml of RIPA buffer, sonicated, centrifuged and the protein concentration estimated. 100 µg of the total protein was separated by electrophoresis on 15% SDS PAGE and transferred to nitrocellulose membrane at 25V for 16 h. The membrane was then stained with Ponceau stain to check the integrity of the transferred proteins and then blocked with 5% skim milk for 2h at room temperature. The membrane was then probed with primary antibody (immune serum) for 1h followed by washes with TBS and TBS-T. It was then incubated with anti-rabbit HRP tagged secondary antibody followed by washes with TBS and TBS-T each for 10 min. At the end of washing, the membrane was developed using chemiluminescence kit.

Immunolocalization

Wistar rats aged 90 days were dissected to collect the testes, spermatozoa and epididymides. Testes were fixed by immersing in Bouin’s solution for 6 h at 37°C followed by washing in 70% ethanol until it destained completely before proceeding with embedding in paraffin wax. Epididymides were fixed by immersing in 4% paraformaldehyde for 12 h and then washed in PBS before block preparation and embedding.

Serial sections (five microns) of testes and epididymides were preheated to 60°C for 5 min followed by xylene wash for 10 min. They were then washed in gradient alcohol (70-100%) followed by phosphate buffered saline (PBS) wash each for 10 min. Antigen retrieval was
carried out by heating the slides in 10 mM citrate buffer pH 6.5 for 12 min in an oven. They were then washed with PBS and permeabilized by incubating in PBS containing 1% triton X-100 (PBST) for 15 min. Tissue sections were blocked with 10% goat serum for 45 min at 37°C followed by incubation with immune serum (1:500 dilution) for 1 h 30 min at 37°C or at 4°C overnight. The slides were then washed in PBS thrice and incubated with anti-rabbit antibody tagged with TRITC or FITC for 1 h followed by three washes with PBS for 10 min each. 4', 6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus. The sections were then mounted with antifade and stored at 4°C in dark. Images were taken using confocal microscope(Khobarekar et al. 2007)(Khobarekar et al. 2007). In the case of spermatozoa, smears on glass slides were prepared and air dried. They were then permeabilized with PBST, blocked with 10% goat serum and processed in a similar way as that of tissue sections.

**Circular Dichroism**

Circular dichroism was performed for recombinant LYZL proteins using Jasco J-810 spectropolarimeter. Quartz cell with a path length of 0.2 cm was loaded with 200 µl of 0.1µg/µl protein and scanned in the far UV region (180-260 nm). Three scans at a scan speed of 50 nm/min were accumulated and the polarimetry data was collected for every 1 nm. The data thus collected was used for calculating mean residue ellipticity (MRE) and the spectrum was plotted (Sreerama & Woody 2004). In addition, the spectra of appropriate blank solution, 10 mM phosphate buffer was subtracted from the spectrum of the protein. Percentage of secondary structure elements were calculated using K2D3 tool of Dichroweb (Louis-Jeune et al. 2011).
Muramidase Assay

The muramidase assay is based on the cleavage of \( \beta \)-glycosidic bond between N-acetyl muramid and N-acetyl glucosamine (Markart et al. 2004). Reduction in O.D at 450 nm is observed as the glycosidic bonds are broken. The muramidase activity of recombinant rat LYZL proteins was determined. Briefly, recombinant rat LYZL protein was incubated with 2 ml of *M. lysodeikticus* cells in 50 mM KH\(_2\)PO\(_4\)-NaOH buffer, pH 7.0, and the decrease in turbidity was monitored at 450 nm in a spectrophotometer for every 60 min until 6 h. Change in O.D (\( \Delta \) O.D) was calculated by subtracting the final O.D from initial O.D. Muramidase activity was expressed as \( \Delta \) O.D. Lysozyme was used as a positive control.

Isopeptidase Assay

The isopeptidase activity assay is based on the cleavage of L-\( \gamma \)-glutamine-p-nitroanilide (L-\( \gamma \)-Glu-pNA) to produce p-nitroanilide (pNA), which exhibits absorbance at 405 nm (Takeshita et al. 2003). Recombinant rat LYZL proteins were added to the reaction mixture containing 1.75 mM L-\( \gamma \)-Glu-pNA in 0.05 M 3-(N-morpholino) propane sulfonic acid (MOPS) buffer, pH 7, containing 0.01M NaCl and the formation of pNA was monitored spectrophotometrically at 405 nm for every 1 h until 6 h. Activity was expressed as \( \Delta \) O.D. Lysozyme was used as positive control.

Antibacterial Assay

Colony forming units (CFU) assay was employed to test the antibacterial activity (Yenugu et al. 2003). Briefly, overnight cultures of *E. coli* XL-1 blue were grown to mid-log phase (\( A_{600} = 0.4 - 0.5 \)) and diluted with 10 mM sodium phosphate buffer (pH 7.4). Approximately 2 X 10\(^6\) CFU/ml of bacteria was incubated at 37°C with 10–100 \( \mu \)g/ml of the recombinant LYZL protein and aliquots of the assay mixture were taken at 30, 60, 90 and
120 min after the start of incubation. The assay mixtures were serially diluted with 10 mM sodium phosphate buffer (pH 7.4) and 100 μl of each was spread on a Luria–Bertani agar plate and incubated at 37°C overnight to allow full colony development. The resulting colonies were hand counted and plotted as log CFU/ml. Lysozyme was used as a positive control.

**Scanning Electron Microscopy**

*E. coli* were treated with recombinant LYZL protein for 120 min and the bacterial cells were fixed in Karnovks’s fixative solution (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1M phosphate buffer) for overnight at 4°C. They were then serially washed with graded alcohol (30% to 100%) for dehydration and finally suspended in acetone before embedding on carbon tape. The samples were then coated with gold and then observed under scanning electron microscope (Yenugu et al. 2004). Lysozyme was used as a positive control.

**Membrane potential measurement**

The effect of LYZL protein on the bacterial membrane potential and permeability was determined by using DiOC<sub>2</sub>(3) and TO-PRO-3 respectively (Novo et al. 2000). DiOC<sub>2</sub>(3) emits green fluorescence as a single molecule, which varies with cell size and is independent of membrane potential. The red fluorescence of DiOC<sub>2</sub>(3) is due to dye aggregation and depends on both size and membrane potential. Therefore the ratio of red to green is attained to measure the membrane potential. TO-PRO-3 is a DNA binding dye and is impermeable to live cells. Due to its ability to enter into membrane compromised cells it is used as dead cell indicator. The dead cells are eliminated when measuring the fluorescence of DiOC<sub>2</sub>(3). *E. coli* grown to mid log phase were diluted in 10 mM sodium phosphate buffer pH 7.4 to a final concentration of 10<sup>6</sup> to 10<sup>7</sup>cells / ml and then treated with 100 μg of recombinant LYZL protein or 15 μM of the bacterial membrane potential
disruptor carbonyl cyanide m-chlorophenyl hydrazine (CCCP) for 2 h at 37 °C in orbital shaker. The cells were then washed in 10 mM phosphate buffer, pH 7.4 followed by incubation with 30 μM DiOC$_2$(3) and 100 nM TO-PRO-3 for 5 min at room temperature. At the end of incubation, bacterial cells were washed and analysed in a flow cytometer (BD LSR Fortessa). The far red fluorescence of TO-PRO-3 is measured in the PerCP-Cy5-5A. The green and red fluorescence of dye DiOC$_2$(3) was measured in FITC and PE-Texas Red-A channel respectively.

**Peptidoglycan binding assay**

96 well plates coated with 40 μg/ml peptidoglycan (PGN) (50μl) or hyaluronan were incubated at 37°C and at 60°C for overnight and 30 min respectively. The plates were then blocked with 1 mg/ml BSA for 2 h and varying concentrations of the recombinant LYZL protein was added to the wells and incubated for 3 h. The wells were then washed 4 times with PBS-T (PBS with 0.1% Tween-20), followed by sequential incubation with primary antibody (1:1000) against the LYZL protein being tested and HRP conjugated secondary antibody (1:10000). After thorough washing, O-Phenylenediamine (OPD) was used to measure the amount of antibody bound to the protein complex and the binding efficiency is measured in terms of ELISA index (EI). ELISA index is calculated by dividing the average O.D of test samples with average O.D of control samples (Wang et al. 2011). Lysozyme was used as a positive control.

**Hyaluronidase activity assay**

0.8% hyaluronan in 300 mM phosphate buffer (pH 7.4) was mixed with melted agarose (0.8%). 100 μl of the gel was dispersed into each well of a microtitre plate. After solidification 50 μl phosphate buffer (pH 7.4) containing varying concentrations of recombinant LYZL protein was added to each well and incubated for 17 h. At the end of the
incubation, solutions were removed and the hyaluronan was precipitated by adding 100 µl of 10% cetyl pyridinium chloride and incubating for 30 min at 37°C. The turbidity developed was read at 595 nm (Handwaskar et al. 2007). Hyaluronidase activity was measured in terms of decrease in turbidity (or O.D). Hyaluronidase was used as a positive control.

**Free radical scavenging assay**

This assay is based on the ability of 1,1-diphenyl-2-picrylhydrazyl (DPPH) to undergo reduction due to the presence of an odd electron, and thus exhibits a strong absorption maximum at 517 nm (You et al. 2010). As this electron becomes paired off in the presence of a hydrogen donor, a free radical scavenging antioxidant, the absorption capacity decreases, resulting in decolorization. 0.04% of 1, 1 diphenyl-2-picryl hydrazyl (DPPH) was dissolved in methanol. To 100 µl of DPPH solution, varying concentrations of recombinant LYZL protein was added and incubated at room temperature for 30 min. Free radical scavenging capacity was assessed by measuring the discoloration of DPPH at 517 nm. Lysozyme was used as a positive control.

**Statistical Analysis**

Statistical analysis were performed using ANOVA and Student’s t-test available in Sigma Plot software (SPSS Inc., Chicago, IL, USA). Values shown are mean ± SD.
RESULTS

Lyzl gene expression

Using gene specific primers, Lyzl1, Lyzl3, Lyzl4, Lyzl5, Lyzl6 and Lyzl7 expression was analysed in various rat tissues. Since the full length sequence of Lyzl4 was not reported it was amplified, sequenced and submitted to GenBank, NCBI and was given the accession no. NM_001246183.1. Among the male reproductive tract tissues, Lyzl genes were expressed only in the testis (Figure 2.1). However, Lyzl7 expression was detected in the cauda along with testis. Further, analysis using cDNA obtained from non-reproductive tissues and female reproductive tissues indicated that Lyzl 3, 5 and 6 transcripts are confined only to the testis. Lyzl1, 4 and 7 were found to be expressed in non-reproductive tissues as well (Figure 2.1).

Figure 2.1: Expression of Lyzl genes in the male reproductive and non-reproductive tissues. RNA isolated from different tissues of rats were reverse transcribed and used for gene specific PCR. Gapdh was used as internal control. Cp-caput, Co-corpus, Cd-cauda, T-testes, Sv-seminal vesicle, P-prostate, B-Brain, H-Heart, Lu-Lungs, Li-Liver, K-Kidney, Sp-Spleen, O-Ovary, Ut-Uterus, Ce-Cervix, Gapdh-Glyceraldehyde 3 phosphate
Gene expression in the male reproductive tract is under the influence of androgens. To elucidate the influence of androgen variation, PCR analyses for Lyzl genes were carried out using total RNA isolated from the epididymidis and testes of 20–60 day old rats. Though the expression of Lyzl transcripts is absent in the epididymis obtained from the adult rats, it is possible that they may be expressed in the younger rats during postnatal development. In the epididymis, none of the Lyzl genes analysed in this study was expressed at all the ages during development (Figure 2.2). In the testes, the Lyzl transcripts seem to be expressed in all the age groups starting from 30 days (Figure 2.2).

**Figure 2.2:** Developmental regulation of Lyzl genes in epididymidis and testes. RT PCR for Lyzl genes in epididymis and testes collected from rats of different age groups. 10, 20, 30, 40, 50 and 60 are the age of rats in days. cp- caput, cd-cauda. Gapdh was used as the internal control.
Cloning and recombinant protein production

Cloning strategy was planned using Vector NTI program. The coding region of the *Lyzl* genes was cloned into pQE80m vector (Figure 2.3). The vector was obtained as kind gift from Dr. Susan Hall, University of North Carolina, U.S.A. The vector contains a T5 promoter followed by ribosomal binding site and a 6X histidine polylinker which codes for N-terminal 6X histidine tag. The coding region is cloned next to the polylinker by making use of the multiple cloning sites (MCS). The vector contains stop codons in all the three frames besides the beta lactamase (*bla*) gene, which confers ampicillin resistance. Details of the restriction sites used, size of the insert and the predicted size of the recombinant proteins are given in table 8. After ligation, plasmids were transformed into *E.coli DH5α* and colonies were checked by colony PCR for presence of the insert using standard vector primers. PCR amplicons corresponding to the size of the respective insert size were observed in majority of the colonies tested (Figure 2.4).

![Figure 2.3: The generalized map of pQE vector (adopted from Qiagen).](image)
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**Figure 2.4:** Cloning of Lyz1 genes into pQE80m vector. Colony PCR using single colonies obtained after transforming E.coli DH5α with plasmids containing Lyz11-7 inserts. Arrow indicates the expected amplicon size.

PCR amplicons from the positive clones were amplified and sequencing analyses confirmed the presence of the insert in proper orientation and in frame with the 6X His-tag. Plasmids isolated from positive clones were propagated into E.coli BL21 to express the recombinant protein by IPTG induction. 6X His-tagged proteins were purified using nickel nitritotriacetate matrix and were found to have minimum contamination as observed on SDS- PAGE and staining with com.maassie brilliant blue (Figure 2.5). Western blotting using anti-His antibody confirmed the identity of the purified proteins basing on the molecular weight (Figure 2.6). In few cases we could observe dimer or trimer formation of the recombinant protein. They were confirmed to be derived from monomers, since they formed monomers upon reduction with tris (2 carboxy) ethyl phosphate (TCEP).
Figure 2.5: Recombinant protein purification of LYZL proteins. Purified recombinant protein fractions were analysed on 15% SDS PAGE. M indicates molecular weight marker. Arrows indicate the purified protein of interest. Numbers indicate the protein fraction number when eluted with buffer E.

Figure 2.6: Western blot analysis of the recombinant LYZL proteins. Immunoblot of recombinant LYZL proteins using anti-His tag antibody. M indicates molecular weight marker. Arrows indicate protein of interest.
Antibody Generation

Recombinant protein was injected intradermally by standard methods and the antibody titer was checked by dot blot. Cross reactivity among the antibodies was checked by Western blotting analysis for all the LYZL proteins with each immune sera. No cross reactivity was observed (Figure 2.7) suggesting that the antibodies generated are specific to the individual LYZL proteins.

Figure 2.7: Cross reactivity of antibodies between recombinant LYZL proteins. Immunoblots showing the specificity of the antibodies generated. All the LYZL proteins were probed with each and every LYZL antibody. L1- Lysozyme-like1, L3- Lysozyme-like3, L4- Lysozyme-like 4, L5- Lysozyme-like 5, L6- Lysozyme-like 6 and L7- Lysozyme-like 7.
Expression of LYZL proteins

Since mRNA expression of the Lyzl genes was found in the rat male reproductive tissues, their translation products (proteins) were also analysed by immunoblotting. LYZL1, 3, 4 and 5 are expressed only in testes (Figure 2.8). LYZL6 is observed in both epididymids and testes. LYZL3, 4 and 6 were detected on sperm, whereas, the other proteins were not. LYZL7 expression was not detected in any of the tissues analysed (Figure 2.8).

\[\begin{array}{cccccccc}
Cp & Cd & T & Sv & P & Spm & Rec & LYZL1 \\
& & & & & & & \\
& & & & & & & LYZL3 \\
& & & & & & & LYZL4 \\
& & & & & & & LYZL5 \\
& & & & & & & LYZL6 \\
& & & & & & & LYZL7 \\
& & & & & & & Actin \\
\end{array}\]

**Figure 2.8:** LYZL protein expression in male reproductive tract. Immunoblotting of LYZL proteins in male reproductive tract tissues. The blots were probed with immune serum specific to each protein. Cp-caput, Cd-cauda, T-testes, Sv-seminal vesicles, P-prostate, Spm-sperm, Rec-recombinant protein.
Immunolocalization of LYZL proteins

LYZL1 protein was localized only in the testes, especially in the germinal epithelium. It was also detected in the head region of spermatozoa obtained from adult rats (Figure 2.9). Similarly, LYZL 3, 4, and 5 (Figure 2.10, 2.11 and 2.12 respectively) were found to be localized in the testes and on the spermatozoa. LYZL3 and 5 are localized to head region of the spermatozoa. LYZL4 expression in the sperm was restricted to tail region (Figure 2.11). LYZL6 expression was detected in both in the epididymidis and testes and also in the head region of the spermatozoa (Figure 2.13). LYZL7 expression was undetectable in all the tissues analysed (Figure 2.14).
Figure 2.9: Immunolocalization of LYZL1. Serial sections of the rat testes and epididymides were incubated with antigen preadsorbed immune serum (peptide control) or immune serum raised against LYZL1, followed by TRITC (Tetramethylrhodamine-5-(and-6)-Isothiocyanate (5(6))) tagged secondary antibody and counter stained with DAPI (4',6-diamidino-2-phenylindole) nuclear stain. Spermatozoa were stained with FITC (fluorescein Isothiocyanate) tagged secondary antibody.
Figure 2.10: Immunolocalization of LYZL3. Serial sections of the rat testes and epididymides were incubated with antigen preadsorbed immune serum (peptide control) or immune serum raised against LYZL3, followed by TRITC (Tetramethylrhodamine-5-(and-6)-Isothiocyanate (5(6)) tagged secondary antibody and counter stained with DAPI (4',6-diamidino-2-phenylindole) nuclear stain. Spermatozoa were stained with FITC (fluorescein Isothiocyanate) tagged secondary antibody.
**Figure 2.11:** Immunolocalization of LYZL4. Serial sections of the rat testes and epididymides were incubated with antigen preadsorbed immune serum (peptide control) or immune serum raised against LYZL4, followed by TRITC (Tetramethylrhodamine-5-(and-6)-Isothiocyanate (5(6)) tagged secondary antibody and counter stained with DAPI (4’, 6-diamidino-2-phenylindole) nuclear stain. Spermatozoa were stained with FITC (fluorescein Isothiocyanate) tagged secondary antibody.
Figure 2.12: Immunolocalization of LYZL5. Serial sections of the rat testes and epididymides were incubated with antigen preadsorbed immune serum (peptide control) or immune serum raised against LYZL5, followed by TRITC (Tetramethylrhodamine-5-(and-6)-Isothiocyanate (5(6))) tagged secondary antibody and counter stained with DAPI (4',6-diamidino-2-phenylindole) nuclear stain. Spermatozoa were stained with FITC (fluorescein Isothiocyanate) tagged secondary
Figure 2.13: Immunolocalization of LYZL6. Serial sections of the rat testes and epididymides were incubated with antigen preadsorbed immune serum (peptide control) or immune serum raised against LYZL6, followed by TRITC-Tetramethylrhodamine-5-(and-6)-Isothiocyanate (5(6)) tagged secondary antibody and counter stained with DAPI (4',6-diamidino-2-phenylindole) nuclear stain. Spermatozoa were stained with FITC (fluorescein Isothiocyanate) tagged secondary antibody.
Figure 2.13: Immunolocalization of LYZL7. Serial sections of the rat testes and epididymides were incubated with antigen preadsorbed immune serum (peptide control) or immune serum raised against LYZL7, followed by TRITC- Tetramethylrhodamine-5- (and-6)-Isothiocyanate (5(6)) tagged secondary antibody and counter stained with DAPI (4,6-diamidino-2-phenylindole) nuclear stain. Spermatozoa were stained with FITC (fluorescein Isothiocyanate) tagged secondary antibody.
The tissue specific mRNA and protein expression analysed by RT-PCR, Western blotting and immunofluorescence correlated to a large extent (Table 9). The presence of LYZL6 protein in the epididymidis (though the mRNA was not detected by RT-PCR) could be due to carry over of this protein by the moving spermatozoa. The absence of LYZL7 protein in all the tissues suggests that its mRNA may not be translated though it is detected by RT-PCR in the testes. The discrepancies within Western blotting and IHC results with regard to the expression of LYZL proteins on spermatozoa could be due to difference in the detection range of the two methods. Altogether these results suggest that the LYZL proteins are expressed predominantly in testes and also on spermatozoa.

**Table 9: Summary of the mRNA and protein expression pattern**

<table>
<thead>
<tr>
<th>LYZL proteins</th>
<th>mRNA Expression (PCR)</th>
<th>Protein Expression (Western Blotting)</th>
<th>Temporal Localization (IHC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cp</td>
<td>Cd</td>
<td>T</td>
</tr>
<tr>
<td>LYZL1</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>LYZL3</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>LYZL4</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>LYZL5</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>LYZL6</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>LYZL7</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Cp-caput, Cd-cauda, T-testes, Spm-sperm. + and – indicates presence and absence respectively.*
Secondary structure analysis

Circular dichroism was performed to understand the folding of the recombinant LYZL proteins. Mean residue ellipticity (MRE) was calculated by using θ values (machine reading), molecular weight and concentration of the recombinant protein and were plotted against the wavelength. The CD spectra of recombinant LYZL proteins (Figure 2.15) show a peak at 210 nm which is characteristic of a α-helical protein. Further, MRE values for each recombinant protein when tested using K2D3 (secondary structure analysis program) showed that these proteins contain α-helix pattern (Table 10) in their structure.

Table 10: Secondary structure measurement of LYZL proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>α-helix</th>
<th>β-sheet</th>
<th>Random coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>LYZL1</td>
<td>87.41%</td>
<td>0.21%</td>
<td>12.38%</td>
</tr>
<tr>
<td>LYZL3</td>
<td>43.1%</td>
<td>31.9%</td>
<td>25.0%</td>
</tr>
<tr>
<td>LYZL4</td>
<td>54.6%</td>
<td>2.63%</td>
<td>42.77%</td>
</tr>
<tr>
<td>LYZL5</td>
<td>60.95%</td>
<td>0.19%</td>
<td>38.86%</td>
</tr>
<tr>
<td>LYZL6</td>
<td>65.89%</td>
<td>1.05%</td>
<td>33.06%</td>
</tr>
<tr>
<td>LYZL7</td>
<td>23.39%</td>
<td>24.55%</td>
<td>52.06%</td>
</tr>
</tbody>
</table>

LYZL proteins exhibit homology with lysozyme and conserve the lactalbumin domain and the 8 cysteine motifs. It is possible that they may exhibit muramidase and isopeptidase activities.
**Figure 2.15:** Circular dichroism spectra of recombinant LYZL proteins measured in terms of mean residue ellipticity (MRE).
Muramidase assay

Only LYZL1 and LYZL6 proteins exhibited muramidase activity whereas the remaining LYZL proteins did not show any activity (Figure 2.16). The activity exhibited was concentration dependent and was comparable to the positive control, lysozyme.

Isopeptidase assay

Among the LYZL proteins that were tested, only LYZL1 and LYZL6 displayed isopeptidase activity in a concentration dependent manner, whereas LYZL3, 4, 5 and 7 did not exhibit any isopeptidase activity (Figure 2.17).

Antibacterial assay

Colony forming units (CFU) assay was employed to test the antibacterial activity of LYZL proteins. LYZL1 and LYZL6 exhibited bacterial killing activity, whereas the remaining proteins failed to decrease bacterial count (Figure 2.18). This may be due to absence of the active site in these proteins.
Figure 2.16: Muramidase activity of LYZL proteins. Varying concentrations (1, 5 and 10 µM) of recombinant LYZL proteins were incubated with *M. lysodeikticus* and the O.D monitored at 450nm. Lysozyme was used as a positive control. Values shown are mean ± SD.
**Figure 2.16:** Muramidase activity of LYZL proteins. Varying concentrations (1, 5 and 10 µM) of recombinant LYZL proteins were incubated with *M. lysodeikticus* and the O.D monitored at 450nm. Lysozyme (1µM) was used as a positive control. Values shown are mean ± SD.
Figure 2.17: Isopeptidase activity of recombinant LYZL proteins. Varying concentrations of (1, 5 and 10 µM) of recombinant LYZL proteins were incubated L-γ-Glu-pNA and the O.D monitored at 405nm. Lysozyme (1µM) was used as positive control. Values shown are mean ± SD.
Scanning Electron Microscopy

*E. coli* treated with recombinant LYZL1 and LYZL6 were observed under electron microscope to study the morphological changes caused by these proteins. PBS treated *E. coli* cells show normal smooth surface (Figure 2.19) whereas the LYZL1 and LYZL6 treated cells display rough cell surface with membrane blebbing. In addition, release of cytosolic content of the bacterial cells was observed. The actions of these proteins are similar to that exhibited by lysozyme.

![Figure 2.19](image)

**Figure 2.19:** Effect of recombinant LYZL1 and LYZL6 on the morphology of *E. coli.*

Scanning electron micrographs of *E. coli* treated with 100 µg/ml recombinant LYZL proteins for 2 h. Lysozyme (10 µg/ml) was used as positive control.
Measurement of membrane potential and Permeability

The membrane potential and permeability of the bacterial cells treated with recombinant LYZL proteins was measured using DiOC$_2$(3). Figure 2.20A shows the measurement of DiOC$_2$(3) fluorescence in FITC-A (green) and PE-Texas Red-A (red) channel. The mean fluorescence intensity on PE Texas Red-A channels denotes the aggregation of the dye due to increased membrane potential. CCCP treatment caused decrease in membrane potential thereby decreased mean fluorescence intensity in PE-Texas Red-A channel. Green fluorescence is the measure of cell size to detect aggregation. CCCP treatment did not cause aggregation of bacterial cells which is indicated by the mean fluorescence intensity in FITC-A channel. Treatment of cells with recombinant LYZL1 and 6 caused increase in green fluorescence showing that they possibly tend to aggregate bacterial cells. Normalized ratio between the red and green fluorescence shows the membrane potential independent of cell size. Addition of recombinant LYZL1 or 6 protein to E. coli resulted in decreased ratio of red/green fluorescence in comparison to phosphate buffer treated bacterial cells (Figure 2.20B), suggesting clump formation and also change in membrane potential due to addition of these proteins. The bacterial cells treated with lysozyme also showed a change in the membrane potential similar to CCCP. TOPRO-3fluorescence is measured in PerCP-Cy5-5A channel. Increase in mean fluorescence intensity denotes increase in the membrane permeability.
Figure 2.20: Dye based membrane potential measurement in E. coli treated with recombinant LYZL proteins using flow cytometry. A- MFI measurements of the cells in FITC, PE-Texas Red A and PerCP-Cy5-5-A channels. B- Membrane potential measured in terms of ratio of mean fluorescence intensity of red/green.
Peptidoglycan binding ability

LYZL domain, which has the catalytic cleft and is responsible for binding to cell wall component was found to be present in all the rat LYZL proteins. We observed that though all LYZL proteins possess the domain, only LYZL1 and LYZL6 show antimicrobial activity. Hence, analysing the binding efficiency of the LYZL proteins with the bacterial cell wall components may help in understanding the differential antibacterial ability. As anticipated, lysozyme displayed a concentration dependent peptidoglycan binding ability. LYZL1 and LYZL6 had higher peptidoglycan binding ability than LYZL3, 4, 5 and 7 which may be due to presence of active site in LYZL1 and 6. However, the binding ability of all the LYZL proteins was significantly less than lysozyme at all the concentrations tested.

**Figure 2.21:** ELISA based peptidoglycan binding assay. 40 µg/ml peptidoglycan coated plate was incubated with 0.25, 0.5, 0.75 and 1µM of the recombinant proteins. Affinity of protein bound to peptidoglycan was measured in terms of colour produced during development after probing with corresponding primary and secondary antibody. Values shown are mean ± SD. *, #, ¥ and ¶ indicates p<0.05 compared to the corresponding concentration of lysozyme.
Hyaluronan binding ability

Lysozyme exhibited hyaluronan binding in a dose dependent manner which may be due to chemical similarity between hyaluronan and peptidoglycan. Among the LYZL proteins tested, LYZL3 had the highest hyaluronan binding ability followed by LYZL4, LYZL5, LYZL1 and LYZL7. LYZL7 displayed hyaluronan binding similar to that of lysozyme. LYZL6 had the least hyaluronan binding ability.

Figure 2.22: ELISA based hyaluronan binding assay. 40 µg/ml hyaluronan coated into the wells of a microtitre plate was incubated with 0.25, 0.5, 0.75 and 1µM of the recombinant protein. Protein binding to peptidoglycan was measured by ELISA based colour detection. ELISA index was calculated by subtracting the average O.D of negative control and dividing the resultant by the negative control O.D. Values shown are mean ± SD. *, #, ¥ and ¶ indicates p<0.05 compared to the corresponding concentrations of lysozyme.
Hyaluronidase activity

Hyaluronidase used as a positive control caused the clearance of cetyl pyridinium chloride resulting in decrease of O.D at 595 nm. Surprisingly, though LYZL proteins had hyaluronan binding ability, none of them exhibited hyaluronidase activity at all the concentrations tested (Figure 2.23).

**Figure 2.23:** Hyaluronidase activity of LYZL proteins. Hyaluronan mixed agarose incubated with different concentrations of recombinant LYZL proteins. The amount of cetyl pyridinium chloride precipitate cleared is a measure of hyaluronidase activity. Hyaluronidase was used as positive control. Values shown are mean ± SD.
**Free radical scavenging activity**

Lysozyme exhibited potent free radical scavenging activity, which is evident by the discoloration of DPPH and thereby decrease in the O.D at 517 nm. Except for LYZL5 and LYZL6, all other LYZL proteins caused a decrease in the O.D of DPPH at 517 nm in a dose dependent manner. Among them LYZL4 had the highest antioxidant potential.

![Free radical scavenging assay. DPPH solution was incubated with varying concentrations of recombinant LYZL protein and the decrease in O.D was measured at 517 nm. Lysozyme was used a control. Radical scavenging activity was expressed in terms of percentage. Values shown are mean ± SD. *, #, ¥ and ¶ indicates p<0.05 compared to the corresponding concentration of lysozyme.](image_url)
**DISCUSSION**

In the present study we report the expression pattern of rat \textit{Lyzl} transcripts and proteins. This is the first comprehensive study undertaken to analyse the expression of rat \textit{Lyzl} mRNA and protein. Some of the \textit{Lyzl} mRNA transcripts were found to be expressed only in testes in the male reproductive tract. Such an exclusive expression in the testis suggests a role for these proteins in spermatogenesis. In addition to their expression in testis LYZL1, 4 and 7 are expressed in other non-reproductive tissues suggesting that these proteins may have roles beyond reproduction. Human LYZL4 transcripts were detected in the testes and pancreas similar to the expression pattern observed in this study (Zhang et al. 2005). The expression of mouse \textit{Lyzl} genes in testes and epididymidis was also reported (Sun et al. 2011). Further LYZL4 was detected in brain and lungs in addition to testes and epididymidis in mouse (Wei et al. 2013). Our results indicate the expression of \textit{Lyzl} in brain, lungs, kidney, ovary and uterus in addition of testes and epididymidis. Our results are more or less similar to that observed in earlier reports. However there are variations in the tissue expression pattern of these genes in different species indicating a possible variation in functional role in different species.

Developmental regulation of a wide variety of genes due to the fluctuations of androgens at various stages in the male reproductive system has been studied extensively (Rodríguez et al. 2001). Androgen levels in the rat epididymis decline from birth until 20 days but remain at a substantial level of approximately 10 ng/g tissue (35 nM) until approximately 40 days when the levels begin to increase to that of the adult, between 15–20 ng/g. Serum testosterone levels in the young rat remain low and do not begin to increase to adult levels until 35–40 days of age (Wei et al. 2013)(Wei et al. 2013). \textit{Lyzl} mRNA transcripts were not detected in the epididymides obtained from 20–60 day old rats. It is possible that their expression pattern is not androgen dependent in this organ system. Testicular androgen
variation during development in the rat was reported to be significantly different from the epididymis. A steady increase in testosterone levels occurs in the rete testis of 30-130 day old rats (Harris & Bartke 1981). In this study, the presence of *LyzII*, 3, 4, 5, 6 and 7 mRNA transcripts was observed in the testes starting from 30 day post natal development. The expression pattern of *LyzI* transcripts analysed in this study seem to correlate with the minimal androgen levels from day 20 to day 40 and increased androgen in the adult, suggesting that *LyzI* gene expression may be androgen dependent during development in the testis. Androgen dependent expression of *LyzI4* during development was reported in the mouse testis (Wei et al. 2013). Further studies are required to determine the molecular mechanisms that operate in controlling the expression of *LyzI* transcripts during development.

The general properties of the recombinant proteins (sequence, molecular weight, pI) correlated well with the predicted properties. In previous reports expression of mRNA transcripts of *LyzI* genes was reported. This is the first study to report the LYZL proteins expression pattern in male reproductive tract by immunolocalization and Western blotting. Localization of LYZL proteins in growing spermatids and in the germinal epithelium indicates that they may have role in spermatogenesis. LYZL4 was found to be localised in tail portion of mouse (Sun et al. 2011). Similar observation was made in case of LYZL6 (Wei et al. 2013). Our results are in consistent with earlier reports. Though some of the proteins are testis specific, they were detected in epididymis also. This could be due to the movement of these proteins along with luminal fluid from testis to epididymis.

Epididymal proteins secreted into the lumen play a key role in sperm maturation (Lassere et al. 2001). For example human PH34 which is secreted by the epididymal epithelium is added to sperm surface and is required for fertilization (Boué et al. 1996). Moreover, certain men with idiopathic infertility show decreased amount of P34H proteins in the seminal
plasma along with fertilization failure, suggesting that this protein can be used as marker for sperm maturation (Boué & Sullivan 1996). Besides this, some of epididymal proteins are known to exhibit potent antimicrobial activity, thereby forming important components of male reproductive tract innate immunity. Human epididymal protein (HE2) is shown to possess potent antimicrobial activity (Yenugu et al. 2004). BIN1b which belongs to the defensin family is secreted in the epididymis and is proven to play important role in sperm maturation and motility, besides antibacterial activity (Guo et al. 2009).

Lysozyme, because of its ability to cleave the glycosidic bond of peptidoglycan, displays potent antimicrobial activity (Ibrahim et al. 2001). Human semen contains lysozyme and is shown to have a positive role on viscosity of the semen (Mendeluk et al. 1997). In this study, we demonstrated that LYZL1 and 6 display potent antibacterial activity against *E. coli*. The antibacterial activity of LYZL proteins was demonstrated in other species. For example, human LYZL6 was found to be a potent antibacterial protein (Wei et al. 2013). LYZL3, 4, 5 and 7 did not display antibacterial activity. This could be due to the lack of essential amino acids in the active site. The human c-type lysozyme SLLP1, was non-bacteriolytic similar to rat LYZL3, 4, 5 and 7 (Mandal et al. 2003). Substrate binding assays also indicate that only LYZL1 and 6 exhibit higher affinity to bind peptidoglycan in comparison with the remaining proteins.

Similar trend was observed in muramidase and isopeptidase assays. These properties can be attributed to the presence of active site residues in LYZL1 and 6. Except LYZL5 and 6, all the LYZL proteins exhibit free radical scavenging activity. The free radical scavenging activity of lysozymes is attributed to the disulphide bonds in these proteins (Memarpour-yazdi et al. 2011). The hyaluronan binding ability of the recombinant LYZL proteins shows the multiple functions played by these proteins. To conclude this part of the study, we report
that rat LYZL proteins are predominantly expressed in the male reproductive tract and they are biochemically similar to that of lysozyme.

REFERENCES


