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Original Article

Lyzl4, a novel mouse sperm-related protein, is involved in fertilization

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The role of Chicken-type (c-type) lysozyme, a prototype lysozyme, in immunity has been characterized in many organisms. In this study, we cloned a novel c-type lysozyme-like gene, *Lyzl4*, which was located on mouse chromosome 9F4 and encoded 145 amino acids with a putative signal peptide and a protease cleavage site. The mature recombinant Lyzl4 protein expressed in yeast did not show the bacteriolytic activity. Sequence alignment analysis demonstrated that 3 of the 20 invariant residues in c-type lysozymes were changed in Lyzl4. One of the 'changed' amino acids (D52G) is located in the catalytic domain. *Lyzl4* mRNA was selectively expressed in testis and epididymis in adult mice, with varying expression level across different developmental stages. High level of Lyzl4 protein was found on the spermatozoa of acrosomal region and principal piece of tail. Immuno-neutralization of Lyzl4 protein in spermatozoa with its specific antibody significantly decreased *in vitro* fertilization percentage in a dose-dependent manner, suggesting that Lyzl4 might be important for fertilization.

Keywords Lyzl4; spermatozoa; fertilization; gene; cloning

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Introduction

Lysozymes that are a group of proteins with bacteriolytic activity, are widely distributed in nature, and have been found in a variety of species, including phages, fungi, plants, invertebrates, birds, and mammals [1,2]. Based on their amino acid sequence and biological origin, lysozymes are classified into six families: chicken-type (c-type), goose-type (g-type), invertebrate-type (I-type), phage, bacterial, and plant lysozymes [2]. C-type lysozymes are widely distributed in insects as well as vertebrates [2–4]. The c-type lysozymes hydrolyze the β-1, 4 glycosidic bonds of peptidoglycan in bacterial cell walls [1,5]. Four c-type lysozyme genes (*LYZL2*, *LYZL4*, *LYZL6,* and *SPACA3*) have been cloned from human male reproductive systems [6]. They are all highly expressed in the testis or epididymis. The *SPACA3* gene encodes a sperm lysozyme-like protein SLLP1. Antisera to SLLP1 can block the sperm–egg binding in hamster egg penetration assay, indicating a possible role of SLLP1 in sperm–egg binding and fertilization [7]. But the function of the remaining three genes is still not clear.

In the current study, the full-length cDNA of mouse Lyzl4 was cloned based on the sequence of human Lyzl4, and the specific anti-mouse Lyzl4 antibody, as well as the recombinant Lyzl4 protein, was prepared. The localization and expression patterns of Lyzl4 mRNA and Lyzl4 protein in mouse were subsequently determined. Furthermore, the bacteriolytic activity of the rLyzl4 protein and the effect of anti-Lyzl4 antibody on *in vitro* fertilization were also examined, with a view to preliminarily explore the functions of Lyzl4 protein played in mouse reproduction.

Materials and Methods

Animals and samples preparation

All mice were obtained from Shanghai Research Center for Model Organisms (Shanghai, China). They were housed in a pathogen-free facility and maintained in a controlled environment (21°C–24°C, 12 h light–dark periods). The brain, heart, lung, thymus, liver, spleen, kidney, stomach, intestine, testis, epididymis, and muscle of C57BL/6J mice were harvested for extraction of total RNA and protein. All animal studies conducted were reviewed and approved by the Institutional Animal Care and Use Committee of Tongji University.

cDNA and protein sequence analysis

Using the amino acid sequence of human LYZL4 as query sequence, a predicted mouse orthologue (NP_081191) was

found in the NCBI database. Based on mRNA sequence (NM_026915), we designed the following primers to amplify a fragment of cDNA in mouse testis library: 5′-GCACC CGAGTGGCCCTGCCTCACTTGCC-3′ (forward); 5′-GG CAAGTGAGGCAGGGCCACTCGGGTGC-3′ (reverse). The polymerase chain reaction (PCR) product was cloned into the pMD19-T vector (Takara, Dalian, China). The sequence of positive clones was aligned to the predicted gene sequence. Motif analysis of Lyzl4 was performed at http://ca.expasy.org/prosite/. Signal peptide and cleavage site were predicted by utilizing the SignalP at http://www.cbs.dtu.dk/services/SignalP/. Clustal W was used for multiple protein sequence alignment at http://www.ebi.ac.uk/Tools/clustalw2/, and the result was edited by the Jalview program [8].

RT-PCR analysis

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, USA). Reverse transcription was performed by using random primers and moloney murine leukemia virus reverse transcriptase (Promega, Madison, USA). Quantitative PCR analyses were performed on an ABI 7000 Taqman system (Applied Biosystems, Foster City, USA). Conditions for PCR amplifications were as follows: initial denaturation at 95°C for 2 min; followed by 40 cycles of 10 s denaturation at 95°C; 20 s annealing at 60°C; and 25 s extension at 72°C. The sequences of the primers were: mouse *Actin*: 5′-TACCCAGGCATTGCTGACAGG-3′ (forward), 5′-ACT TGCGGTGCACGATGGA-3′ (reverse); *Lyzl4*: 5′-GGGTG TGCCTGGCTTATTTTGAGA-3′ (forward), 5′-GGAGCT GGCCAGTGGTGAGGTAA-3′ (reverse).

Production and purification of anti-Lyzl4 sera

The 129 bp cDNA sequence of Lyzl4 was inserted into a modified pGEX-3X vector (*Not*I and *Kpn*I cloning sites was inserted between *Bam*HI and *Eco*RI; Amersham Biosciences, Piscataway, USA). The resulting plasmid encoded a recombinant fusion protein [glutathione S-transferase (GST)−Lyzl4] that contained the Lyzl4 peptide from amino acid 29 to 71. *Escherichia coli* (*E.coli*) BL21(DE3) cells transformed with the pGEX-3X−Lyzl4 plasmid were inoculated into 200 ml of 2×YTA media containing 100 μg/ml ampicillin. The cells were incubated at 37°C until reaching an optical density of 1.0 at 600 nm, and then induced with 0.05 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma, St Louis, USA) for 3 h at 30°C. The purification of GST−Lyzl4 protein was performed using a GSTrap™ 1 ml column (Amersham Biosciences). The polyclonal antisera against GST−Lylz4 were obtained according to a standard immunization protocol [9]. Two rabbits were immunized three times with one milligram of the antigen on days 1, 3, and 28. Antisera were harvested on the 35th day. The titers were assayed by enzyme-linked immunosorbent assay (ELISA) using the purified GST−Lyzl4 protein as the coating antigen.

To remove antibodies against GST, the antisera were subjected to an immunoaffinity chromatography column containing cyanogen bromide (CNBr)-activated Sepharose 4B coupled to GST and *E.coli* total protein. For the production of *E.coli* total protein and GST protein, *E.coli* BL21(DE3) transformed with pGEX-3X plasmid was incubated and induced with IPTG. The total protein was extracted after the cells were harvested and sonicated. The coupling of sepharose and total protein was performed using CNBr-activated Sepharose 4B (Amersham Biosciences).

Cell culture and transfection

Human embryonic kidney cells (HEK293T) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml of streptomycin. Cells were incubated at 37°C under an atmosphere of 5% CO_2. Transient transfection of HEK293T cells was done with Lipofectamine 2000 (Invitrogen). After transfection, the cells were incubated 48 h before further study.

Western blot analysis

Protein samples were separated on 15% sodium dodecyl sulfate (SDS)−ployacrylamide gels (PAGE) and transferred onto polyvinyl difluoride membranes (Amersham Biosciences). The membranes were blocked with tris-buffered saline tween-20 buffer containing 5% fat-free milk powder for 1 h at room temperature, and then incubated overnight at 4°C with the first antibody against Lyzl4 (1:1000), GFP (1:1000; Santa Cruz, Santa Cruz, USA), β-actin (1:10,000; KangChen Biotech, Shanghai, China) or GAPDH (1:10,000; KangChen Biotech). After washing, membranes were incubated with an appropriate horseradiah peroxidase conjugated secondary antibodies (1:10,000; Sigma) or fluorescent conjugated secondary antibody for 1 h (1:10,000; LI-COR Biosciences, Lincoln, USA). The protein bands of interest were analyzed with an enhancement of eletrochemiluminescence advanced western blotting detection Kit (Amersham Biosciences) or Odyssey Infrared Imaging System (LI-COR Biosciences).

Production and purification of recombinant Lyzl4 protein (rLyzl4) from yeast

A DNA fragment coding mature Lyzl4 peptide of 126 amino acids and 6×Histidine tags were inserted into the pPIC9K (Invitrogen) by using 5′ *Eco*RI and 3′ *Not*I restriction sites. The sequences of the primers were: 5′-GGG GAATTCAAGTCCATCTTGGGGCGCTGC-3′ (forward); 5′-GGGGCGGCCGCTTAGTGGTGGTGGTGGTGGTGC AGATC-3′ (reverse). Twenty micrograms of the resulting

plasmid (pPIC9K–Lyzl4) was linearized and transformed into *Pichia pastoris* strain *GS115* by electroporation. The transformed cells were screened on yeast extract peptone dextrose medium (YPD) plates containing geneticin at the concentration of 0.5, 1, 2, or 4 mg/ml. A clone selected from 4 mg/ml geneticin YPD plates was inoculated in 2 ml YPD medium for 48 h at 30°C, and then transferred into 1 L YPD medium and cultured for 48 h. The cells were harvested by centrifugation at 7700 g for 10 min and resuspended in east extract peptone medium. To induce expression, methanol was added in east extract peptone medium to a final concentration of 1% every 24 h. After 3 days of induction, the supernatant was collected by centrifugation. The pH was adjusted to 5.0 with acetic acid. The supernatant was loaded onto a cation-exchange chromatography column (SP-Sepharose^{TM} Fast Flow, GE Healthcare, Bethesda, USA). Sample was eluted with 10 mM imidazole in phosphate buffer. Eluted fractions were subjected to HisTrap^{TM} HP 1 ml column (GE Healthcare), and the resin was eluted with a linear gradient of elution buffer (2.5%–100%, 20 mM phosphate, 400 mM imidazole, pH 8.0). Protein concentration was determined by a bicinchoninic acid method and the purity was analyzed by 15% SDS–PAGE.

Assay of bacteriolytic activity of rLyzl4

Bacteriolytic activity was measured with a turbimetric assay and a lysoplate assay as described [10,11] with some modifications. The turbimetric assay measures the decrease of the substrate (0.2 mg/ml dried *Micrococcus lysodeikticus*) in 0.1 M phosphate (pH 7.5) or citrate buffer (pH 4.4) after incubation with rLyzl4 or Chicken Lysozyme (1 mg/ml; Sigma). In the lysoplate assay measures, heat-killed *M. lysodeikticus* in 0.1 M phosphate (pH 7.5) or citrate buffer (pH 4.4) were added to molten 1.5% lysogeny broth agar at a final concentration of 0.4 mg/ml and poured into Petri dishes. rLyzl4 (40 μg) or chicken lysozyme (10 μg) was added into wells pre-punched in the plate. The plates were incubated at 37°C overnight before measuring the clearance zone.

Indirect immunofluorescence detection of Lyzl4 in spermatozoa

Indirect immunofluorescence detection was performed as described [12] with some modifications. Mouse sperm was collected from cauda epididymis, washed in PBS, air-dried on polylysine-coated slides, and fixed in 4% paraformaldehyde for 15 min. Non-specific binding was blocked with 10% normal goat serum. The sperm was then incubated with anti-Lyzl4 serum (1:100) or pre-immune rabbit serum (1:100) overnight at 4°C. After washing, sperm was incubated with fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody (1:500; Sigma). The slides were washed and incubated with PI (1:1000; Sigma) for 10 min. After washing, the sperms were observed under an Olympus BX-52 microscope.

In vitro fertilization

In vitro fertilization was performed as described previously [13,14] with some modifications. Oocytes were obtained from hybrid F1 mice (C57BL/6J×CBA) by superovulation. Sperm was collected from the vasa deferentia and cauda epididymis of sexually mature male mice (C57BL/6J). The sperm was diluted to a concentration of 2×10^5 sperm/ml in a volume of 50 μl human tubal fluid drops under paraffin oil (Sigma; embryo tested), and incubated with anti-Lyzl4 serum or pre-immune serum in a humidified tissue culture incubator (37°C, 5% CO_2 in air) for 1 h. Before the experiment, the antiserum and pre-immune serum were decomplemented at 56°C for 30 min. After the incubation, the cumulus masses were placed in the drops (average 20 eggs in one drop, at least 3 drops in one group), and then cultured overnight at the same condition. Fertilization was examined next morning: two-cell embryos were considered as fertilized; one-cell oocytes were considered as unfertilized.

Results

Cloning of *Lyzl4* cDNA and sequence analysis

Using the human LZYL4 protein sequence as a BLASTP query to search in the protein database, an ortholog protein of human LYZL4 in mouse was found, the product of prediction gene of *Lyzl4*. Based on the predicted mRNA sequence (NM_026915), primers were designed to identify whether the *Lyzl4* was expressed in the mouse. Realtime RT-PCR was performed with different mouse tissue RNA. A 495 bp fragment including the entire open reading frame (ORF) was amplified from the mouse testis library (**Fig. 1**).

Lyzl4, spanning ~64 kb, was located on mouse chromosome 9F4. Two *Lyzl4* transcripts encoding the same protein sequence were predicted by Ensembl. All the introns involved in both transcripts conformed to the GT/AG rule of splice junction [15]. The predicted full-length *Lyzl4* cDNA consisted of a 438 bp ORF that encoded an acidic protein of 145 amino acids. Sequence analysis indicated a signal peptide at position 1–19 and a cleavage site at 19–20, indicating Lzyl4 is a secretory protein. Cleavage of the signal peptide leads to a mature protein consisting of 127 amino acids with a molecular weight of 14.1 kDa and an isoelectric point (PI) of 5.65. A strong signature sequence of alpha-lactalbumin/lysozyme C family was found by using the Scanprosit at http://ca.expasy.org/tools/scanprosite/.

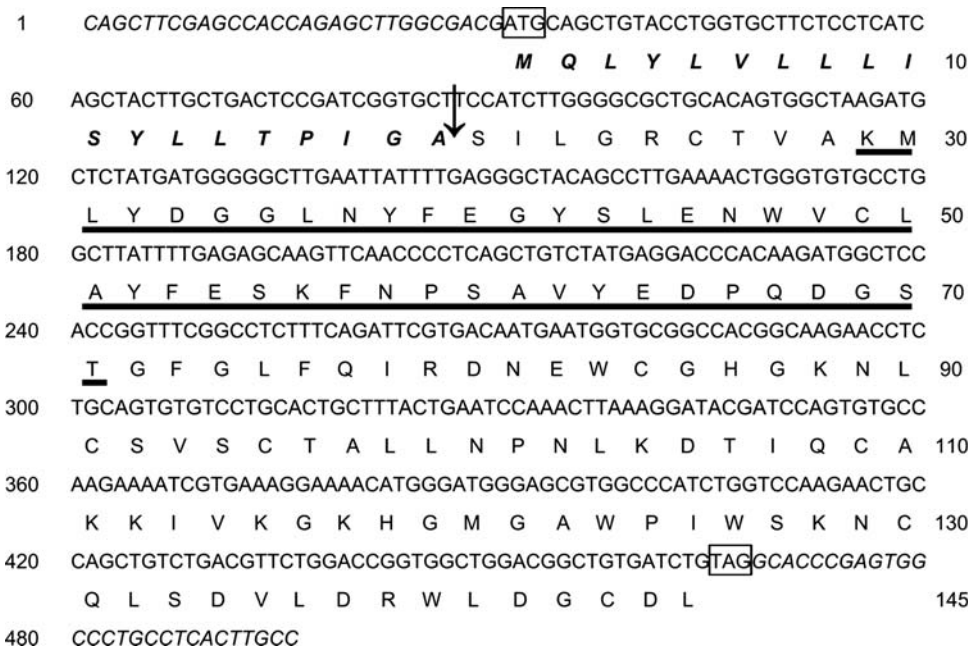

Figure 1 Nucleotide sequences of cloned *Lyzl4* cDNA and deduced amino acid sequences of Lyzl4 The upper line indicated the cDNA sequence (number at left) and the untranslated regions were indicated in italics. The initial and terminal codons were boxed. The putative signal peptide was indicated in bold and italics and the position of cleavage site was shown as arrow. The peptide used to raising antibody was indicated as underline.

Alignment of Lyzl4 with other c-Lysozyme/lactalbumin family members and phylogenetic relationship analysis

Alignment of the deduced mature amino acid sequences with other c-lysozyme/lactalbumin family members showed that the mouse Lyzl4 is 74% identical to human LYZL4 and 30% identical to human alpha-lactalbumin. On average, LYZL4 is 40% identical to the five typical c-type lysozymes (**Fig. 2**). Among the 20 invariant amino acid residues in typical c-type lysozyme, 17 were found to be conserved in Lzyl4. One of the variant residues was D52 that was one of two key residues for the lysozyme bacteriolytic activity and overall three-dimensional structure of active-site cleft. Four of six potential substrate binding sites were different from other c-type lysozymes in Lyzl4. Five deletions located between positions 70 and 74 in the Lyzl4 sequence were also found by multiple alignments.

Production of antibody against Lyzl4

cDNA fragment encoding the Lyzl4 between 29 and 71 incorporated with a GST tag was expressed in *E.coli*. As shown in **Fig. 3(A)**, a 31.6 kDa band (GST–Lyzl4) was induced and existed in the supernatant of bacteria lysis. After purification, a prominent GST–Lyzl4 band was observed, but another band of less intensity was also noted. The extra band was recognized by anti-GST antibody (data not shown) and represents a GST catabolism product in our opinion. The purified GST–Lyzl4 protein was used to raise antisera in rabbits. The titers of antisera were greater than 10^6.

As shown in **Fig. 3(B1)**, the antisera before GST–antigen column purification could detect several bands with total GST–Lyzl4, GST–ZF12 (another GST fusion protein) bacterial protein samples and purified GST–Lyzl4 protein samples. Purified antisera only detected one band with total GST–Lyzl4 bacterial protein samples and purified GST–Lyzl4 protein samples, while no immune reaction with GST–ZF12 protein samples was detected [**Fig. 3(B2)**]. So, the purified antisera only recognized the antigen fragment of Lyzl4. A single band was detected in 293T cells transfected with Lyzl4–enhanced green fluorecence protein plasmid and no band was detected in untransfected 293T cell (data not shown), indicating a high antigen-specificity of the purified anti-Lyzl4 antibody.

Bacteriolytic activity assay of secretory Lyzl4

To get a soluble Lyzl4 with correct folding structure for determining the potential bacteriolytic activity, a DNA fragment encoding mature Lyzl4 protein and six histidine tags was transfected into the methylotrophic yeast, *P. pastoris*. After G418 screening, a stable integrated strain was selected. After methanol induction, a 14.9 kDa band was observed. The secreted Lyzl4 was purified from the media by Ni^{2+} chelation affinity chromatography [**Fig. 4(A)**]. The specificity of induced and purified protein was verified with antisera of Lyzl4 by Western blot [**Fig. 4(B)**].

Both turbimetric and lysoplate assay did not reveal any bacteriolytic activity (data not shown).

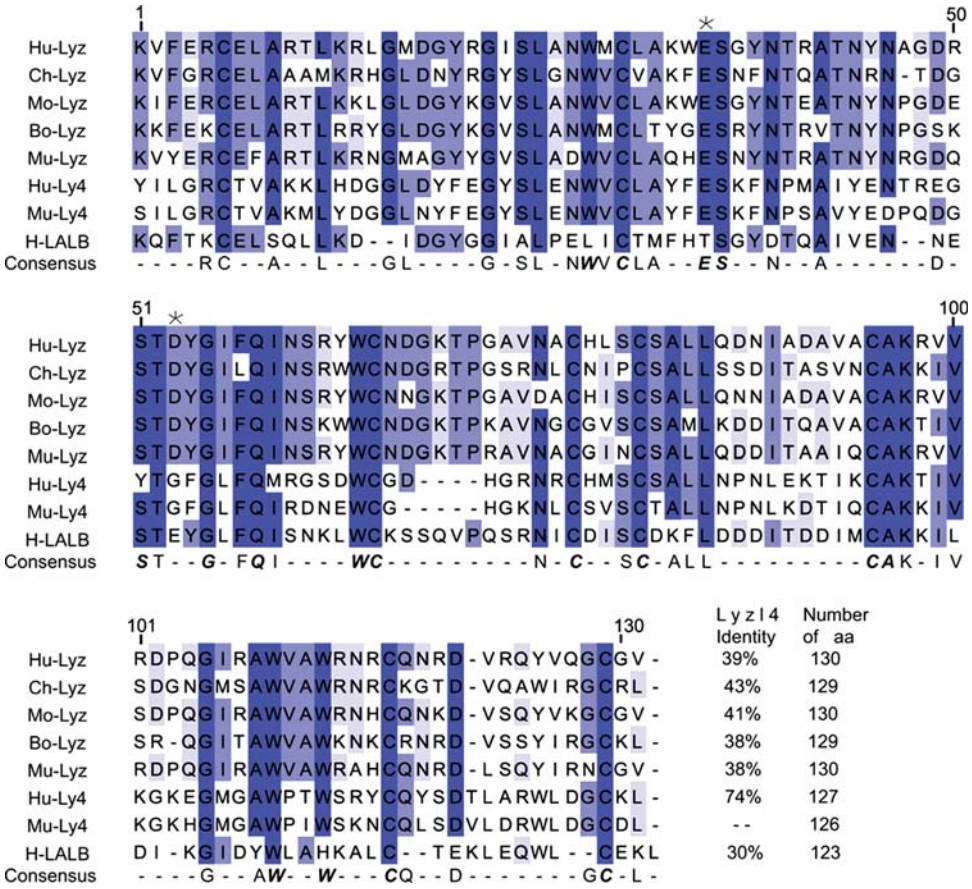

Figure 2 Alignment of deduced amion acid sequences of mature Lyzl4 with other c-type lysozymes and human alpha-lactalbumin Gaps that were introduced into the sequences were shown as dashes. Residues that were shaded showed identical at least four out of eight proteins, and Lyzl4 residues shown identical with these shaded residues were used to as the consensus sequence. The conserved residues of Lyzl4 in 20 invariant residues of c-type lysozymes were indicated in bold and italic letters in the consensus sequences. Asterisks indicated the key amino acids involved in catalytic activity of c-type lysozymes. The residue number of alignment proteins and percent identity of Lyzl4 with these proteins were shown at the end of these sequences. Hu-Lyz, human lysozyme (accession: NP_000230); Ch-Lyz, Chicken egg white lysozyme (accession: NP_990612.1); Mo-Lyz, monkey stomach lysozyme (accession: P67977); Bo-Lyz, bovine intestinal lysozyme (accession: NP_001007806.1); Mu-Lyz, mouse lysozyme (accession: NP_059068.1); Hu-Ly4, human Lysozyme-like 4 (accession: NP_653235); Mu-Ly4, mouse lysozyme-like 4 (accession: NP_081191); H-LALB, human alpha lactalbumin (accession: NP_002280).

Expression pattern of *Lyzl4* mRNA in different tissues and growth stage in mouse testis

To determine the expression pattern of *Lyzl4* mRNA, total RNA from different tissues were isolated and RT-PCR was performed. The result revealed that *Lyzl4* was selectively expressed in testis and, to a lesser degree, in epididymis [**Fig. 5(A)**]. To investigate whether the expression of Lyzl4 is growth stage dependent, we analyzed *Lyzl4* mRNA expression in different growth stages of testis by Realtime-PCR. RT-PCR experiment failed to detect *Lyzl4* expression in the testis of 2-weeks-old neonates, while the expression reached a peak level at 12 weeks. After that, the level gradually decreased as the age increased [**Fig. 5(B)**].

Localization of Lyzl4 in mouse

To determine the distribution of Lyzl4 in mouse, immunoblot analysis was performed with different tissue and cell samples. Western blot detected a 14.1 kDa band in sperm extract, but not in the mRNA expression tissues of testis and epididymis or other tissues [**Fig. 5(C)**]. Indirect immunofluorescence staining analysis showed that Lyzl4 was predominantly located on the spermatozoa of acrosomal region and principal piece of tail (**Fig. 6**).

Blocking Lyzl4 inhibited *in vitro* fertilization

To investigate the function of Lyzl4 located in the sperm, an *in vitro* fertilization was performed. The capacitated mouse spermatozoa were pre-incubated with different dilution of Lyzl4 antisera to block the Lyzl4 in spermatozoa, and then were incubated with cumulus-oocyte complexes (average 20 eggs in one drop, at least 3 drops in one group). At 1:200 and 1:100 dilution, Lyzl4 antisera reduced fertilization by 45 and 80%, respectively ($P < 0.001$ and $P < 0.05$ vs. pre-immune sera; **Fig. 7**). Furthermore, the percentage of

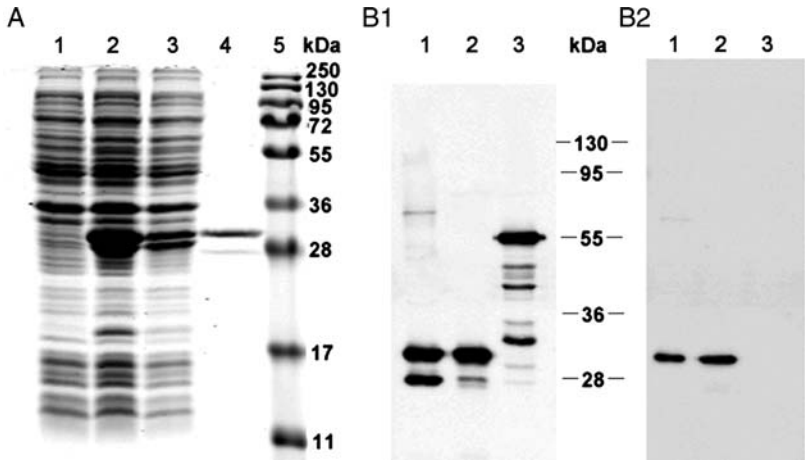

Figure 3 Purification of GST–Lyzl4 and assay of antisera specificity (A) Induction and purification of GST–Lyzl4 in *E.coli* by Coomassie blue staining. From left to right: lane 1, uninduced total *E.coli* protein; lane 2, 3 h induced total *E.coli* protein; lane 3, sonicated supernatant of induced *E.coli*; lane 4, purified GST–Lyzl4 protein; lane 5, molecular weight markers. (B) Specificity of prepurified (B1) and purified (B2) antisera by western blot. From left to right: lane 1, total GST–Lyzl4 *E.coli* protein; lane 2, purified GST–Lyzl4 protein; lane 3, total ZF12GST3 *E.coli* protein (as a GST control).

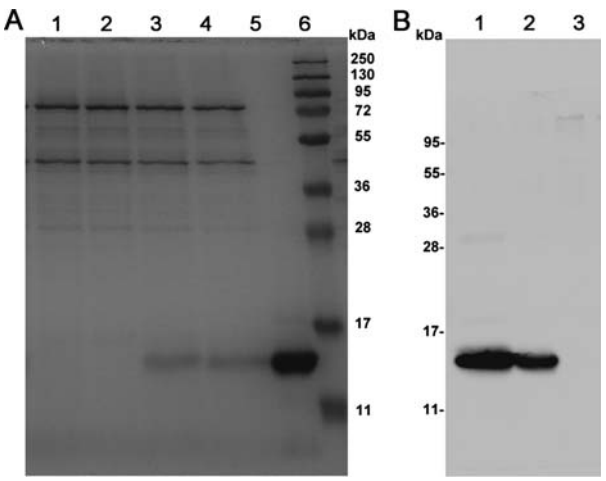

Figure 4 Expression and purification of rLyzl4 from yeast supernatant (A) SDS–PAGE analysis of induced rLyzl4 in cluture media and purified rLyzl4. From left to right: lane 1 and 2, the media of negative control clone (transformed pPIC9K vector) induced for 2 and 3 days; lane 3 and 4, the media of rLyzl4 clone (transformed pPIC9K–Lyzl4) induced for 2 and 3 days; lane 5, the purified rLyzl4; lane 6, molecular weight markers. (B) Western analysis of rLyzl4. From left to right: lane 1, the purified rLyzl4; lane 2, the media of rLyzl4 clone induced for 3 days; lane 3, the media of negative control clone induced for 3 days.

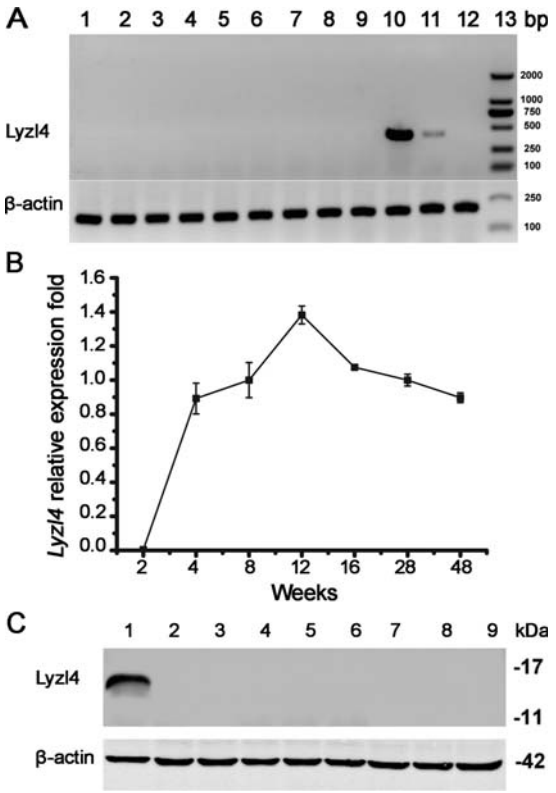

Figure 5 Expression profile of *Lyzl4* gene and Lyzl4 protein (A) RT-PCR analysis of *Lyzl4* expression in different tissues (lanes 1–13: brain, heart, lung, thymus, liver, spleen, kidney, stomach, intestine, testis, epididymis, muscle, and molecular makers). (B) Realtime-PCR analysis of *Lyzl4* expression in mouse testis at different growth stages. (C) Western blot analysis of Lyzl4 in different tissues and spermatozoa (lane1–9: spermatozoa, epididymis, testis, kidney, spleen, liver, lung, heart, and brain).

two-cell embryos was gradually decreased with the raise of the Lyzl4 antisera concentration in immune sera groups.

Discussion

In the present study, we identified an orthologue of human *LYZL4* in mouse. This gene was mainly expressed in the male reproductive system. The expression level varied significantly during postnatal development. Experiments in yeast expression system revealed that mature Lyzl4 lacks bacteriolytic activity. The Lyzl4 protein was located in the spermatozoa of acrosomal region and principal piece of tail. Blocking the Lyzl4 in spermatozoa using antisera inhibited the *in vitro* fertilization.

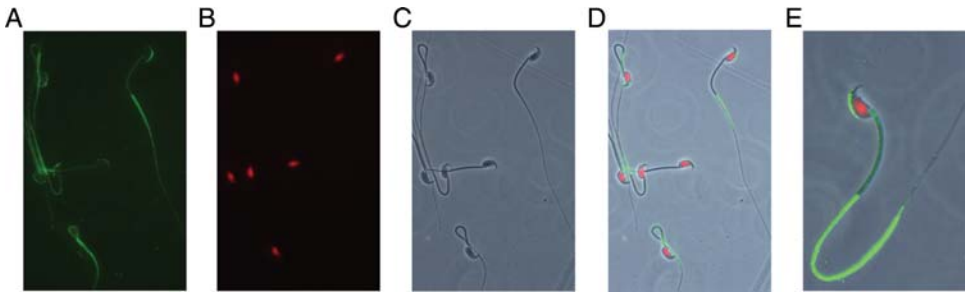

Figure 6 Indirect immunofluorescent localization of Lyzl4 protein in spermatozoa (A) the immunofluorescence of Lyzl4; (B) the PI labeled nuclear; (C) phase contrast view of spermatozoa; (D) the merged photograph of A, B, and C; Magnification 20× for A, B, C, and D; (E) magnified a whole merged spermatozoa photograph.

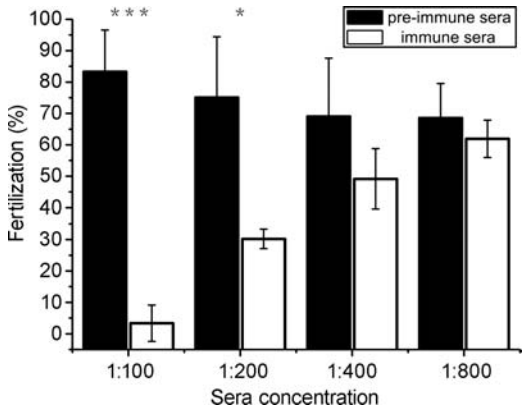

Figure 7 Effect of Lyzl4 antisera on *in vitro* fertilization Data are representative of three independent experiments. Two-cell embryos were scored as fertilization. $*P < 0.05$; $***P < 0.001$.

Lyzl4, a novel c-type lysozyme gene

Based on the deduced protein sequence, Lyzl4 was classified into c-type lysozyme/α-lactalbumin family, although Lyzl4 was considerably different from lysozymes or α-lactalbumins in protein sequence. C-type lysozyme and α-lactalbumin have similar amino acid sequence and dimensional structure, but quite different functions [16,17]. Lysozymes represent a major defense against bacteria by hydrolyzing peptidoglycan in bacterial walls and transglycosylation [18], whereas lactalbumins catalyze the biosynthesis of lactose in the mammary gland [19]. On average, Lyzl4 is 40% identical to the already known classical c-type lysozymes, and 30% identical to human α-lactalbumin. Seventeen out of the 20 (85%) invariant amino acids of c-type lysozymes were conserved in Lyzl4. In contrast, only 13 out of 20 (59%) were identical to that in lactalbumin. C-type lysozyme has a fairly wide tissue distribution, whereas lactalbumine is only expressed in the mammalian milk and colostrums [16]. Our results indicated that *Lyzl4* was selectively expressed in the testis and epididymis. Taken together, we believe Lyzl4 is a c-lysozyme rather than α-lacatalbumin.

Lyzl4, a lysozyme-like protein of non-bacteriolytic activity

Lysozymes hydrolyze glycosidic linkage between the C-1 of *N*-acetylmuramic acid (NAM) and the C-4 of *N*-acetylglucosamine (NAG) in the polymer of NAM and NAG of bacterial cell walls [2]. Although Lyzl4 apparently belongs to the c-type lysozyme family, no bacteriolytic activity was observed. Based on the protein sequence alignment, we found that 3 of 20 invariant residues are substituted (D52G, Y53F, and N59R). All three substitutions occur in the 50–76 flexible hydrophilic area; one is an essential catalytic reside (52-Asp). SLLP1, another lysozyme-like protein encoded by human *SPACA3* gene, also have substitution at the two essential catalytic residues and lack bacteriolytic activity [7]. We therefore believe that replacing essential catalytic residue(s) is responsible for the loss of bacteriolytic activity of Lyzl4.

The role of Lyzl4 located in the spermatozoa

In the mammalian testis, undifferentiated germ cells transform into spermatozoa through a complex series of events that include mitosis, meiosis, and cellular differentiation [20]. Spermatozoa acquire the ability to fertilize eggs in epididymis. This process requires the presence of many factors secreted by the epithelium cells, e.g. SLLP1 [7] and HongrES1 [21], as well as direct interaction with epithelium cells of the epididymis [22]. SLLP1 is an intra-acrosomal sperm protein specifically expressed in testis. The presence of SLLP1 in acrosomal of spermatozoa is essential for sperm–egg binding to occur. HongrES1 is a serine proteinase inhibitor protein exclusively expressed in the cauda epididymis, and plays an important role in sperm capacitation. Western blot in our experiment revealed the presence of Lyzl4 in spermatozoa but not on testis or epididymis, the mRNA expression tissues. Indirect immunofluorescence staining showed Lyzl4 is located on the spermatozoa of acrosomal region and principal piece of tail. These results indicated the Lzyl4 might be expressed and secreted in the testis and epididymis, and then becomes concentrated on the spermatozoa.

Blocking Lyzl4 in spermatozoa with antisera inhibited fertilization in our *in vitro* experiment. The Lyzl4 antisera could have achieved this action by any of the following two mechanisms or the combination of two mechanisms.

First, neutralization of the Lyzl4 in the head of spermatozoa could interfere with the recognition of spermatozoa and egg or preclude the undergoing of acrosomal reaction. Alternatively, neutralization of Lyzl4 in the tail could reduce the mobility of spermatozoa. A previous study demonstrated that antisera against SLLP1 (another c-type lysozyme-like protein) could also inhibit fertilization [7]. The human orthologue of *Lyzl4* and *SLLP1*, *LYZL4,* and *SPACA3*, are two of four c-type lysozyme-like genes specifically expressed in human testis/epididymis [6]. The two lysozyme-like proteins share similar genomic organization and protein structure. Results from our study supported the hypothesis that some lysozyme-like proteins might be involved in fertilization. We are currently establishing a *Lyzl4* knockout mouse line to further investigate their *in vivo* action.

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