CHAPTER IV
MATERIALS AND METHODS

1. Screening of a cDNA library with a legumain conserved probe and analysis of the sequences of FgLGMN-1 and FgLGMN-2 isolated from a cDNA library of adult *Fasciola gigantica*

1.1 Production of recombinant LGMN cDNAs

1.1.1 PCR amplification and cloning of highly conserved legumain regions from an adult stage *F. gigantica* cDNA library

Degenerated oligonucleotide primers based on two highly conserved amino acid motifs (NYRHQAD and IYFTDHG) (Table 3) present in the legumain family were used to amplify cDNA fragments from an adult stage *F. gigantica* cDNA library constructed in λTriplEx2 (Meemon *et al*., 2004) by PCR.

The following components were combined in the reaction tube, 1 μl of cDNA template ($1x10^9$ pfu/ml), 37.5 μl of DDW, 5 μl of 10× PCR buffer, 1 μl of 50× dNTPs mix, 1 μl of 5' PCR primer, 1 μl of 3' PCR primer, and 0.5 μl of Taq DNA polymerase enzyme (5 U/μl) (Fermentas Life Sciences, Vilnius, Lithuania). The samples were gently mixed and spun down. PCR was performed using the following conditions: 94°C for 5 minutes, 30 cycles of 94°C for 1 minute, 43°C for 1 minute, and 72°C for 1 minute then 72°C for 5 minutes and hold at 4°C. Samples of the PCR products were analyzed on a 1% agarose gel (containing 0.5 μg of ethidium bromide per one milliliter of agarose gel).

Table 3 Degenerated primers designed for amplification of LGMN conserved regions

Primer	Conserved regions	Degenerated primer sequences
Forward LGMN conserve	NYRHQAD	5'-AAYTAYAGRCAYCARGCNGAY-3'
Reverse LGMN conserve	IYFTDHG	5'-NCCRTGRTCNGTRAARAADAT-3'

The underlined nucleotides indicate: R=AG (meaning, R can be A or G), Y=CT, D=AGT, N=ACGT

1.1.2 Agarose gel electrophoresis

Agarose gels were prepared by adding agarose powder into 0.5× TBE buffer (22.5 mM Tris-base, 22.5 mM boric acid, 1 mM EDTA, pH 8) to the required final concentration, *e.g.* 0.7% for DNA and 1.2% for RNA. In order to completely dissolve the agarose, it was heated in a microwave oven until the solution became clear and homogeneous. The agarose solution was allowed to cool to approximately 50-60°C before ethidium bromide (0.5 μg/ml) was added and mixed with the homogeneous gel solution, then it was poured into the gel tray (Phamacia-biosciences, USA). The gel was allowed to harden for 45 minutes before it was submerged in TBE/ethidium bromide buffer (0.5 μg/ml ethidium bromide in TBE buffer). 10× sample buffer (0.25% bromophenol blue, 0.25% xylenecyanol FF, and 50% glycerol) was added to the DNA sample at 1× final concentration before the sample was loaded to the gel. Electrophoresis was performed at 80 V (~2-8 V/cm of agarose gel) until the bromophenol blue dye front had migrated about 2/3 of the gel length. The nucleic acid pattern on the gel was directly visualized under ultraviolet light.

Correctly sized DNA fragments, representing putative conserved of legumain sequence were excised and purified by a gel extraction kit (Qiagen, Germany). The purified DNA fragments were inserted into the pGEM®-T Easy vector (Promega, USA) in a ligation reaction.

1.1.3 QIAEX II DNA extraction from agarose gel

The DNA band was excised from the agarose gel with a clean, sharp scalpel. The gel slice was weighed in a microcentrifuge tube and then three volumes of buffer QX1 were added to one volume of gel for DNA fragments of 100 bp-4 Kb. The QIAEX II particles were resuspended by vortexing for 30 seconds and then added to the sample of 10-30 μl depending on the amount of DNA sample. The sample was incubated at 50°C for 10 minutes and mixed by vortex every 2 minutes for binding the DNA to QIAEX II particles. The mixture was centrifuged for 30 seconds, at 12,000 ×*g* and the supernatant was carefully removed with a pipette. The pellet was washed with 500 μl of buffer QX1, resuspended by vortex and then the sample was centrifuged again for 30 seconds at 12,000 ×*g*. Traces of supernatant were removed with a pipette. The pellet was washed twice with 500 μl of buffer PE, centrifuged for

30 seconds, at 12,000 ×*g* and traces of supernatant were carefully removed. The pellet was allowed to dry at room temperature until became white.

The DNAs were eluted from QIAEX II particles by adding 20 µl of DDW, resuspending the pellet by vortex and incubated at room temperature for 5 minutes. The suspensions were centrifuged for 30 seconds at 12,000 ×*g* and the supernatant was carefully pipetted into a clean new microcentrifuge tube.

1.2 Cloning the PCR product of highly conserved regions of legumain family and transformation of a host bacterial strain with the recombinant plasmid

1.2.1 Ligation of the PCR product to a pGEM®-T Easy vector

The following components were added in a 1.5 ml reaction tube, 2 µl DNA, 2 µl of pGEM®-T Easy vector (500 ng/µl) (Promega, USA), 1 µl of 10× ligation buffer, 1 µl of ATP (10 mM), 1 µl of T4 DNA Ligase, and 3 µl of DDW. The reaction sample was mixed, briefly spun down and incubated at 4°C overnight. The ligation products were introduced into *E. coli* XL1-blue competent cell (Competent cell preparation, described in section 1.2.2) by transformation.

1.2.2 Preparation and storage of competent *E. coli* cells

The XL1-blue strain *E. coli* was plated on LB agar containing 15 µg/ml of the antibiotic tetracycline and freshly grown for 16-20 hours, at 37°C. The isolated single colony of bacteria was inoculated in 100 ml of LB broth at 37°C and shaken at 300 rpm until OD 0.5 at 600 nm. The flask containing the bacterial suspension was vigorously swirled in a salt-ice bath for 3 minutes to bring the temperature to 0°C. The bacterial cells were harvested by centrifuging at 4,000 ×*g* for 10 minutes at 4°C. After the supernatant was discarded, the pellet was gently resuspended in 20 ml of ice-cold 0.1 M $MgCl_2$ by agitation.

The mixture was again centrifuged at 4,000 ×*g* for 10 minutes at 4°C, the supernatant was then decanted and the pellet was resuspended in 20 ml of ice-cold 0.1 M $CaCl_2$. The cells were kept on ice for 20 minutes, centrifuged at 4,000 ×*g* for 10 minutes at 4°C, and the supernatant was decanted. The cell pellet was gently resuspended in 4.3 ml of 0.1 M $CaCl_2$ and mixed with 7 ml of glycerol. The

competent *E. coli* XL1-blue were dispensed into 100 µl aliquots, shock frozen in liquid nitrogen for 5 minutes, and stored at -80°C.

1.2.3 Transformation of competent *E. coli* XL1-blue with recombinant pGEM®-T Easy plasmids

The 0.1-50 ng of recombinant pGEM®-T Easy plasmid were distributed into a 15 ml prechilled polypropylene tube. The competent cells were thawed on ice, 100 µl of the competent cells were added to the plasmid, and gently mixed. The mixture was incubated on ice for 20 minutes. After that, the tube was heat-shocked at 42°C in a water bath 45 seconds and then the tube was put immediately on ice for 2 minutes. 900 µl SOC medium were added and the tube was incubated at 37°C with shaking at 250 rpm for 1 hour. The suitable dilution (50-200 µl) of the mixture was spread on prewarmed LB agar containing 100 µg/ml ampicillin, 0.5 mM IPTG and 80 µg/ml X-Gal. The plates were incubated at 37°C overnight. Several single isolated white colonies were kept on the new LB agar containing 100 µg/ml ampicillin and subjected to isolate plamids and restriction endonucleases (described in section. 1.2.1)

1.2.4 Plasmid mini purification

The single, isolated bacterial colony containing recombinant pGEM®-T Easy plasmid was picked up and inoculated to 5 ml of LB medium containing 100 µg/ml ampicillin. The mixture was incubated at 37°C overnight with shaking at 250 rpm. On the following day, the bacterial cells were pelleted by centrifugation at 4,000 ×*g* for 10 minutes. The pellet was completely resuspended in 250 µl Buffer P1 (50 mM Tris-Cl, 10 mM EDTA and 100 µg/ml RNase A). 250 µl of Buffer P2 (200 mM NaOH and 1.0% SDS) were added and gently mixed by inverting 6-8 times. After that, the mixture was added to the 350 µl Buffer P3 (3.0 M Potassium acetate, pH 5.5) and mixed immediately by inverting several times. The mixture was subsequently centrifuged at maximum speed (12,000 ×*g*) for 10 minutes.

The supernatant was applied to the QIAprep spin column and centrifuged for 30-60 seconds at 12,000 ×*g*. The column was washed with 750 µl Buffer PE (1.0 M NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol). The plasmid DNA was eluted from

the column with sterile DDW by incubating one minute at room temperature and was then centrifuged for 30-60 seconds at 12,000 ×*g* to collect the eluent in a new sterile microcentrifuge tube. The quality of plasmids was analyzed by 1% agarose gel electrophoresis and then subjected to digest with *Eco*R I restriction enzyme (Fermentas Life Sciences, Vilnius, Lithuania) that has no recognition site within the recombinant fragment. After electrophoresis analysis, the positive clones were collected and used for further experiments.

1.3 Construction and labeling of a legumain conserved probe

1.3.1 Digestion reaction of recombinant pGEM®-T Easy containing fragments of correct size

The recombinant pGEM®-T Easy vector containing corrected fragment was digested with *Eco*R I restriction endonuclease according to 14 μl sterile DDW, 4 μl 10× buffer R+ reaction buffer, 2 μl *Eco*R I restriction enzyme (10 U/μl, Fermentas Life Sciences, Vilnius, Lithuania) and 20 μl of the recombinant pGEM®-T Easy vector. The reaction mixture was mixed, spun down, and incubated at 37°C for 2 hours. The digestion products were analyzed by 1.2% agarose ethidiumbromide gel electrophoresis. The correct size fragments were isolated from agarose gel by DEAE membrane DNA isolation technique (described in section 1.3.2).

1.3.2 DEAE membrane DNA isolation technique

The DEAE membranes (NA45, Schleicher & Schuell, Germany) were cut into strips and equilibrated in EDTA equilibrating buffer (10 mM EDTA, pH 8.0) for 5 minutes. The membranes were incubated later with 0.5 N NaOH for 5 minutes, followed by six times washing in sterile DDW. Membranes can be stored in sterile DDW for weeks.

After electrophoresis separation in a horizontal slab apparatus, a strip of NA45 DEAE membrane was placed in an incision in the agarose gel just ahead of the band of interest. The electrophoresis was continuously run until completed binding of DNA to the membrane (approximately 10 minutes). After electrophoresis, the membranes were thoroughly washed in low-salt buffer (50 mM Tris pH 8.0, 0.15 M NaCl, 10 mM EDTA) at room temperature for removing any residual agarose. The

DNAs were eluted by putting the membranes in a microcentrifuge tube. High salt buffer (50 mM Tris pH 8.0, 1 M NaCl, 10 mM EDTA) was added and DNA was eluted at 65°C for 30 minutes. The buffer was collected in the new microcentrifuge tube and the membranes were eluted again with 200 µl high salt buffer at 65°C for 15 minutes. The buffer was collected, pooled with the first eluate and DNA was precipitated with 80 µl 10 M ammonium acetate and 800 µl prechilled 95% Ethanol. The sample was mixed well and incubated at room temperature for 10 minutes. The mixture was centrifuged 12,000 $\times g$ at room temperature for 20 minutes. After that, the pellet was washed with 400 µl 70% Ethanol, centrifuged at 12,000 $\times g$ at room temperature for 5 minutes and dried at room temperature. The pellet was dissolved in 50 µl sterile DDW. The eluted DNA was checked by electrophoresis.

1.3.3 Labeling of the legumain conserved probe

The isolated cDNA fragment was labeled by using the Hexalabel DNA labeling kit (MBI, Fermentas Life Sciences, Vilnius, Lithuania) and radioactive ^{32}P-dCTP. One hundred nanogram of cDNA templates were denatured by boiling at 100°C for 5 minutes and incubated with hexanucleotide in 5× reaction buffer, Mix C containing dGTP, dATP, dTTP, ^{32}P-dCTP and Klenow fragment (exo$^-$), at 37°C for 5 minutes to allow the synthesis of the newly labeled strands. The reactions were stopped by using 1 µl of 0.5 M EDTA, pH8.0 and the unincorporated dNTP was removed by size-exclusion chromatography.

1.3.4 Purification of Radiolabeled Oligonucleotides by size-exclusion chromatography

A Bio-Gel P-60 column was prepared in a sterile small-size plastic column. Initially, a sterile glass wool was tamped to plug into the bottom of column. A small amount of Tris-SDS chromatography buffer (0.1 M NaCl, 20 mM Tris-HCl pH 7.4, 10 mM EDTA, 50% [v/v] glycerol, 40% (w/v) saturated bromophenol blue) was poured into the column and checked that the buffer flows at a reasonable rate (one drop every few seconds). The Bio-Gel P-60 slurry was filled and allowed to settle down by gravity and form the gel matrix. The column was washed with Tris-SDS chromatography buffer. After that, the ^{32}P radiolabeled oligonucleotide was rapidly

loaded onto the column and then Tris-SDS chromatography buffer was added to the top of the column. A hand-hold minimonitor was used to follow the progress of the radiolabeled oligonucleotide. When the radioactivity starts to elute from the column, the fractions were collected in a microcentrifuge tube until the first peak of radioactivity was eluted from the column. The radioactive labeled probe was immediately used for screening of *Fasciola gigantica* cDNA libraries.

1.4 Immobilization of bacteriophages onto nitrocellulose membrane

1.4.1 Titering the amplified adult stage *F. gigantica* cDNA library

The amplified adult *F. gigantica* cDNA libraries (Meemon, *et al*., 2004) were used for the experiments. The isolated colony from the working stock plate of *E. coli* XL1-Blue was inoculated in 20 ml of LB broth containing 10 mM $MgSO_4$ and 0.2% (w/v) maltose. The bacteria suspension was incubated at 37°C overnight with shaking at 140 rpm until the OD_{600} of the culture reaches 2.0. Bacterial cells were centrifuged at 4,000 ×*g* for 10 minutes, the supernatant was poured off and the pellet was resuspended in 7.5 ml of 10 mM $MgSO_4$. Four LB agar plates containing 10 mM $MgSO_4$ were warmed and dried before using. The phage lysate (library) was prepared by pipetting 10 µl of the library lysate into 1 ml (dilution 1= 1:100) of 1× lambda dilution buffer and transferring 10 µl of dilution 1 into a second tube containing 1 ml of 1× lambda dilution buffer (dilution 2= 1:10,000). Four tubes were prepared as described in Table 4. The tubes were incubated at 37°C for 15 min. Each tube was added to three milliliters of melted (45°C) LB/$MgSO_4$ top agar, quickly mixed and the contents from each tube were poured onto LB/$MgSO_4$ agar plates. The plates were swirled quickly to promote an even distribution of the melted agar and cooled at room temperature for 10 minutes to allow the soft agar to harden. The plates were incubated in the inverted position, at 37°C for at least 6-7 hour. The plaques were counted and the titer (pfu/ml) was calculated as follows:

$$\text{pfu/ml} = \frac{\text{number of plaques} \times \text{dilution factor} \times 10^3\ \mu\text{l/ ml}}{\mu\text{l of diluted phage plated}}$$

A successfully amplified library has to have a very high titer (~10^{10} pfu/ml)

Table 4 Plating dilutions for titering an amplified library

1× lamda tube	1× lamda dilution Buffer	*E.coli* XL-1-Blue overnight culture	Phage dilution 2
1	100 µl	200 µl	5 µl
2	100 µl	200 µl	10 µl
3	100 µl	200 µl	20 µl
4 (control)	100 µl	200 µl	0 µl

1.4.2 Plaque formation

600 µl of overnight growth *E. coli* XL1- Blue (prepared as described in section 1.4.1) were mixed with the phage lysate (library) at final concentration 5×10^4 pfu/ml. The mixture tubes were homogenized by inverting and incubated at 37°C for 15 minutes. 6.5 ml of melted (45°C) LB/$MgSO_4$ top agar were added, quickly mixed and poured onto LB/$MgSO_4$ agar plates. The plates were swirled quickly after pouring and cooled at room temperature for 20 minutes to allow the soft agar to harden. The plates were incubated at 37°C for at least 6-7 hours.

1.4.3 Transferring of phages, Isolation of viral DNA and Immobilization of viral DNA

The agar plate containing plaques were kept at 4°C for 1 hour. The 132 mm. round Nytran nylon membranes (Schleicher&Schuell, Germany) were placed on the surface of the infected plates. The orientation of the membranes was marked and the pahges were allowed to transfer for approximately 5 minutes. The membranes were placed by facing up the plaques on a filter paper saturated with denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 5 minutes at room temperature. The membranes were then transferred to one sheet of filter paper saturated with neutralizing solution (0.5 M Tris, pH 8.0, 1.5 M NaCl) for 5 minutes and 2× SSC (0.3 M NaCl, 30 mM sodium citrate pH 7.0) for 5 minutes, respectively. The membranes were completely dried at room temperature on the filter paper. The viral DNAs were fixed by baking under vacuum condition at 80°C for 1 hour. The membranes were kept in a dry and light protected place at room temperature until use.

1.5 Hybridization of FgLGMN-1 and FgLGMN-2 probes onto nitrocellulose membrane and detection

1.5.1 High density plaque hybridization

The nylon membranes were equilibrated in 30 ml of hybridization buffer (50% formamide, 5× SSC, 20 mM Phosphate buffer, 5× Denhardt's solution, 100 mg/ml heat-denatured Herring sperm DNA, 0.5% SDS) and incubated at 42°C in shaking water bath for 1 hour. ^{32}P radiolabeled oligonucleotide probes were denatured at 95°C for 5 minutes and then added to the prehybridizaton buffer (not directly pipette

onto the membranes). The membranes were incubated at 42°C in a shaking water bath, overnight. On the following day, the hybridization buffers were removed and kept at -20°C for further work. The membranes were washed in wash buffer 1 (2× SSC, 0.1% SDS) at 42°C for 30 minutes and transferred to wash buffer 2 (0.1× SSC, 0.1% SDS) for 1 hour.

1.5.2 Autoradiography

The membranes were placed by facing up the plaques on a filter paper for removing excess moisture and then wrapped in plastic wrap to keep remaining moisture inside. The membranes were put and fixed in the cassettes and exposed to X-ray films in the dark room. The cassettes were tightly closed and kept in -80°C, overnight. The autoradiograph was developed in the dark room by soaking the X-ray films in developing solution (Kodak) for 3 minutes. The films were then briefly washed in tap water and soaked again in fixing solution (Kodak) for 3 minutes. The fixing solution was removed out by washing with tap water. The films were dried at room temperature and then compared to the high density plates. Positive plaques were collected and equilibrated in 1 ml 1× lambda dilution buffer with 100 µl chloroform and used for low density plaque hybridization.

1.5.3 Low density plaque hybridization

To isolated single plaques, the low density plaque hybridization was performed by mixing 200 µl of overnight growth *E. coli* XL1- Blue (prepared as described in section 1.4.1) with 1 µl of high density isolated plaques. The mixture tubes were homogenized by inverting and incubated at 37°C for 15 minutes. Three milliliters of melted (45°C) LB/$MgSO_4$ top agar were added quickly, mixed and poured onto LB/$MgSO_4$ agar plates. The plates were swirled quickly after pouring and cooled at room temperature for 20 minutes to allow the soft agar to harden. The plates were incubated at 37°C for at least 6-7 hours. The agar plates containing plaques were used for plaque lift hybridization technique.

1.6 Converting λTriplEx2 phage genome to pTriplEx2 phagemid vector

BM25.8 strain *E. coli* frozen cells from a glycerol stock were recovered by streaking a small portion (~5 μl) of the frozen stock onto LB agar plate containing 50 μg/ml kanamycin and 150 μg/ml chloramphenicol. The plates were incubated at 37°C overnight. A single isolated colony from the working stock plate of *E. coli* BM25.8 was used to inoculate 10 ml of LB/$MgSO_4$ broth. The culture medium was inoculated at 31°C overnight with shaking (150 rpm) until the OD_{600} of the culture reaches 1.1−1.4 and then 100 μl of 1 M $MgCl_2$ was added to 10 ml overnight culture of *E. coli* BM25.8 suspension (10 mM final concentration of $MgCl_2$). Positive plaques from secondary screening plates were picked and placed in 350 μl of 1× lambda dilution buffer. The plaques were mixed and incubated at 37°C for 3-4 hour (alternatively at 4°C overnight). In a 15 ml conical tube, 200 μl of overnight cell culture were combined with 150 μl of eluted positive plaques and incubated at 31°C for 30 minutes without shaking. 1-10 μl of infected cell suspension were spread on an LB agar plate containg 50 μg/ml ampicillin to obtain isolated colonies and grown at 31°C overnight. Several colonies from each clone were obtained and plasmid DNA was prepared separately from each one.

1.7 Purification of plasmid DNA from *E. coli*

1.7.1 Quick preparation of plasmid DNA (Birnboim and Doly, 1979)

The plasmid DNA was used for checking the presence of an insert gene in the pTriplEx2 plasmid. Each colony of plasmid containing *E. coli* was inoculated into 5 ml LB-medium containing 50 μg/ml ampicillin and incubated overnight at 37°C and at 250 rpm. The bacteria were harvested by centrifugation at 4,000 ×*g* for 10 minutes, the pellet was resuspended in 200 μl solution I (25 mM Tris pH 8.0, 50 mM glucose, 10 mM EDTA) and transferred to a 1.5 ml microcentrifuge tube. 400 μl of freshly prepared solution II (0.1 N NaOH, 1%SDS) were added to the mixture and mixed by inverting the tube and subsequently incubated on ice for 5 minutes. After that 300 μl solution III were added to the mixture, mixed by inverting and incubated on ice for 5 minutes, respectively. The sample was centrifuged at 12,000 ×*g* for 5 minutes at room temperature and the supernatant was transferred to a new 1.5 ml microcentrifuge tube.

Isopropanol (2-propanol) was added at 0.6 volume to the tube and mixed by inverting. The mixture was centrifuged at 12,000 ×*g* for 5 minutes, at room temperature and the supernatant was discarded. The pellet was washed with 500 µl of 70% ethanol, centrifuged at 12,000 ×*g* for 2 minutes, at room temperature and dried. The pellet was resuspended in 50 µl of TE buffer. The plasmids were transformed to competent *E. coli* XL1-Blue and then the plasmids were purified. To check the size of insert DNA, several clones of recombinant pTriplEX2 plasmid were digested with restriction endonuclease (*Kpn* I and *Xho* I) at 37°C for 2 hours and analyzed on a 0.7% agarose/ethidium-bromide gel. The bacteria containing plasmids with the proper size of insert gene were collected and further purified for sequencing.

1.7.2 Midi Preparation of plasmid DNA

For sequencing of the insert gene, the plasmid DNAs were purified from bacteria by using JETSTAR plasmid Midi kit (Genomed, Germany). A single, isolated bacterial colony containing a recombinant pTriplEx2 plasmid was cultured in 25 ml LB medium containing 50 µg/ml ampicillin and incubated at 37°C overnight with shaking at 250 rpm. On the following day, the bacteria were harvested by centrifugation at 4,000 ×*g* for 10 minutes. First, the column was equilibrated with 10 ml solution E4 (600 mM NaCl, 100 mM sodium acetate, 0.15% TritonX-100 pH 5.0) before applying the cleared lysate. The harvested bacterial cells were then resuspended in 4 ml solution E1 (50 mM Tris, 10 mM EDTA pH 8.0, 100 µg/ml RNase A) and cells were lysed by adding 4 ml solution E2 (200 mM NaOH, 1.0% SDS), mixed gently by inverting until the lysate appeared to be homogeneous. The mixture was incubated at room temperature for 5 minutes. To neutralize the mixed suspension, 4 ml solution E3 was added and mixed immediately by multiple inverting until a homogeneous suspension is obtained. The cell debris and denatured chromosomal DNA was removed by centrifugation at room temperature at 12,000 ×*g* for 10 minutes. The supernatant was collected and applied to the equilibrated JETSTAR column and the lysate was allowed to run by gravity flow. The column was washed with 10 ml solution E5 (800 mM NaCl, 100 mM sodium acetate pH5.0) twice and the plasmid was eluted with 5 ml solution E6 (1250 mM, 100 mM Tris-HCl pH8.5). The DNA was precipitated with 0.7 volumes of isopropanol and then

centrifuged at 4°C and 12,000 ×*g* for 30 minutes. The pellet of the plasmid DNA was washed with 70% ethanol and centrifuged. The plasmid DNA was dried and dissolved in a suitable volume of sterile DDW. Plasmid concentration was determined by spectrophotometry and analyzed by agarose gel electrophoresis to determine the quality.

1.8 Sequencing and sequence analysis

The purified plasmid DNA isolated from a single-isolated colony from each clone was prepared for sequencing. Full-length cDNAs were identified after sequencing of both DNA strands (MWG, Ebersberg, Germany). Analyses of nucleotide sequences and deduced amino acid sequences were done using EMBOSS 2.8 (Rice *et al*. 2000), ExPaSy and SignalP 3.0 (Bendtsen *et al*. 2004). Server-based searches for homologous sequences were done by BLAST (Altschul *et al*. 1997) through the NCBI server at http://www.ncbi.nlm.nih.gov/BLAST/. Homologous sequences determined in these searches were then received from the GenBank database. The multiple alignment was calculated by ClustalW (Thompson *et al*. 1994) using the BLOSUM62 scoring matrix and phylogenetic tree was reconstructed by bootstrap neighbor-joining tree method (Saitou and Nei, 1987).

2. Characterization of the FgLGMN-1 and FgLGMN-2 genes in various developmental stages with respect to the isotypes and the gene expression

2.1 Preparation of different developmental stages of *F. gigantica*

2.1.1 Metacercariae preparation

To study the whole life cycle of *F. gigantica*, eggs were obtained from bile in gall bladder of naturally infected cattle killed at local abattoir in Pathumthanee province. The eggs were washed several times with 0.85% NSS to get rid of contaminated bile. The eggs were exposed to the light of a lamp for hatching the miracidia. The intermediate snail hosts, *Lymnaea ollula* were infected with miracidia and then maintained in plastic bowls containing fresh circulating water until the cercariae are completely developed. Pieces of plastic sheet were floated on the surface of water, the cercariae released from the snail hosts settled on the sheet where they

were encysted and developed into metacercariae. The metacercaria were obtained by brushing out from plastic sheets under the stereomicroscope and washed with 0.85% NSS and collected in tubes.

2.1.2 Developing juvenile preparation

BALB/c mice were orally infected with metacercariae via the stomach tube. Developing juveniles of *F. gigantica* were obtained from liver of experimental animals at 1st, 2nd, 4th and 6th week after infection (WAI). Blood was collected before the mice were scarified and serum was obtained for further analysis. The juveniles were washed several times with 0.85% NSS and kept in liquid nitrogen for processing in further experiments.

2.1.3 Adult parasite preparation

Adult *F. gigantica* were collected from the liver, bile duct and gall bladder of the naturally infected cattle killed at a local abattoir in Pathumthanee province. The parasites were washed several times with 0.85% NSS and processed for further experiments.

2.2 Isolation of genomic DNA

Frozen adult *F. gigantica* were mixed with a small amount of liquid nitrogen and ground to a powder by using a precooled pestle and mortar. The frozen powder was resuspended in 500 µl of homogenization buffer (30 mM Tris-HCl pH 8.0, 0.1 M NaCl, 10 mM EDTA, 0.5% Triton X-100) and centrifuged at room temperature and 5,000 ×*g* for 5 minutes. The supernatant was discarded and the pellet was resuspended in 500 µl of extraction buffer (0.1 M Tris-HCl pH 8.0, 0.1 M NaCl, 20 mM EDTA), centrifuged at room temperature and 5,000 ×*g* for 2 minutes and resuspended again with 300 µl of extraction buffer. The suspension was incubated with 3 µl of proteinase K (10 mg/ml) (Fermentas Life Sciences, Vilnius, Lithuania) and 15 µl SDS (20%) for 1 hour at 50°C. To extract the proteins, an equal volume phenol-chloroform was added twice, mixed and extracted one time with chloroform. The mixture was centrifuged at 12,000 ×*g* for 5 minutes and the supernatant always carefully transferred to a fresh tube. The solution was treated with RNase A (Fermentas Life

Sciences, Vilnius, Lithuania) to remove RNA by adding 10 µl of RNase A (10 mg/ml) and incubated for 1 hour at 40°C. The phenol-chloroform and chloroform steps were done to remove RNase A as before. The genomic DNA solution was transferred to a fresh tube and DNA was precipitated by adding 2.5 volume ethanol (kept -20°C) and 0.1 volume 3 M sodium acetate. The mixture was mixed, centrifuged at 12,000 ×*g* for 20 minutes and the supernatant was discarded. The pellet was washed with 70% ethanol, dried and resuspended in 100 ml TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

2.3 Isolation of total RNA

Metacercariae, 1-, 2-, 4-, and 6-week old juveniles, and adult *F. gigantica* were homogenized in 1 ml of TRIzol Reagent (Invitrogen, USA) per 50-100 mg by use of a tissue homogenizer (IKA, Germany). To remove insoluble material from the homogenate, the sample was centrifuged at 12,000 ×*g* for 10 minutes at 4°C and the cleared homogenate solution was transferred to a fresh tube and incubated for 5 minutes at room temperature. 0.2 volume of chloroform was added per one volume of TRIzol reagent, the tubes were shaken vigorously by hand for 15 seconds and incubated at room temperature for 2 to 3 minutes. The sample was centrifuged at no more than 12,000 ×*g* for 15 minutes at 4°C. A colorless aqueous upper phase was transferred to a fresh tube and RNA was then precipitated from the aqueous phase by mixing with 0.5 volume isopropyl alcohol per one volume of TRIzol reagent used for the initial homogenization. The sample was incubated at room temperature for 10 minutes and centrifuged at no more than 12,000 ×*g* for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed once with one volume of 75% ethanol. The sample was mixed by vortexing and centrifuged not more than 7,500 ×*g* for 5 minutes at 4°C. At the end of the procedure, the pellet was briefly dried and dissolved with DEPC-treated H_2O. The RNA was determined concentration by spectrophotometry at wavelength 260 nm.

2.4 Construction, labeling and efficiency analysis of FgLGMN-1 and FgLGMN-2 DNA probes

2.4.1 Synthesis of FgLGMN-1 and FgLGMN-2 DNA DIG-labeled hybridization probes by using PCR-DIG labeling mix

FgLGMN-1 was amplified with forward primer (5'-GCATGCATGCACTTCTGTTTGCTG-3') and reverse primer (5'-CTGCAGGATCACTCACAAGCGGTG-3'). FgLGMN-2 with forward primer (5'-GGATCCATGTTATCCATGCAAATTTTATTC-3') and reverse primer (5'-CTGCAGTCAAACACAAACATCATG-3'). The templates, the recombinant pTriplEx2 plasmids containing FgLGMN-1 and -2 fragments were diluted with sterile DDW to 1-100 pg/μl. The following components were combined in the reaction tube, 1 μl of recombinant plasmids solution, 66 μl of sterile DDW, 10 μl of 10× PCR buffer, 10 μl $MgCl_2$, 10 μl of PCR DIG labeling mix (Roche, Penzberg, Germany), 1 μl of forward primer (Operon, Cologne, Germany), 1 μl of reverse primer (Operon, Cologne, Germany), and 1 μl of Taq DNA polymerase enzyme (5 U/μl). The reactions were gently mixed and spun down. PCR was performed using the following conditions: 94°C for 5 minutes, 30 cycles of 94°C for 1 minute, 45°C for 1 minute, and 72°C for 1 minute then 72°C for 8 minutes and hold at 10°C. The synthesized PCR-DIG labeled hybridization probes were analyzed on 1% agarose gel. After the specific PCR products were visible under UV light, the PCR-DIG hybridization probes were determined for their efficiency.

2.4.2 Determination of labeling efficiency

The recombinant pTriplEx2 plasmid containing FgLGMN-1 and -2 fragments were serial diluted with sterile DDW to 1 μg/μl, 0.1 μg/μl, 0.01 μg/μl, 1 ng/μl, 0.1 ng/μl, 0.01 ng/μl, 1 pg/μl, 0.1 pg/μl, 0.01 pg/μl. One milliliter of dilution series were spotted on a nylon membrane and hybridized with the DIG-labeled PCR probes using 10 μl of PCR reaction in 1 ml hybridization solution at 50°C. The DIG-labels were detected with the DIG Nucleic Acid Detection Kit (Roche, Germany).

2.4.3 Cross-hybridization analysis between FgLGMN-1 and FgLGMN-2

The recombinant pTriplEx2 plasmids containing the FgLGMN-1 and -2 fragments were cut with *Kpn* I and *Xho* I restriction endonucleases (Fermentas Life Sciences, Vilnius, Lithuania). Then, the fragments were size-separated on a 1% agarose gel and transferred to a nylon membrane. The FgLGMN-1 fragment was hybridized with the FgLGMN-2 DIG-labeled PCR probe and the FgLGMN-2 fragment was hybridized with the FgLGMN-1 DIG-labeled PCR probe at 50°C. The DIG-labels were detected with the DIG Nucleic Acid Detection Kit (Roche, Germany). Hybridization and immunological detection were described according to the procedure of Southern hybridization analysis (section 2.5).

2.5 Southern hybridization analysis

2.5.1 Southern transfer

The concentration of genomic DNA was approximately determined by comparing the intensity to uncut λ phage DNA in a 0.7% agarose gel under UV light. 10 μg of genomic DNA was fragmented by digestion with *Pae* I (*Sph* I), *Pst* I (Fermentas Life Sciences, Vilnius, Lithuania) and combined *Sph* I/*Pst* I and then incubated at 37°C for 4 hours. The DNA fragments were separated on a 0.7% agarose gel in 0.5× TBE buffer including 0.5 μg/ml ethidium bromide at 25 V, overnight. The gel was photographed under UV light and the distance indicated by a ruler. After that, the agarose gel containing fragmented genomic DNA was denatured by soaking the gel in denaturing buffer (3 M NaCl, 0.5 N NaOH) for 30 minutes and changed to neutralizing buffer (0.5 M Tris-HCl pH 7.0, 1.5 M NaCl) twice, for 20 minutes each. The gel was immerged in 20× SSC transfer buffer (3.0 M NaCl, 0.3 M sodium citrate pH 7.0) for 30 minutes. To prepare the membrane, the nylon membrane (Hybond N, amersham, USA) was floated in DW, allowing the membrane to wet entirely and then soaked in 20× SSC.

To transfer the DNA onto the nylon membrane, the capillary transfer method was performed. Two pieces of 0.39 mm gel blot paper (Hybond, blotting paper, Amersham, USA) were cut 4-6 inches larger than the gel, saturated with transfer buffer and placed on the plastic plate of the gel blotting set. The plate was placed in the transfer buffer reservoir container by both ends of the gel blot paper draped into a

buffer reservoir. The agarose gel was placed on top of the gel blot paper, then the prepared membrane, followed by two pieces of gel blot paper cut to fit the gel was placed on the gel. A stack of paper towels was placed on top of the gel blot paper. Enough transfer buffer was added to the container to completely saturate the gel blot paper on the lowest layer. A weight was placed on the top of the stacking paper towels. The DNA was allowed to transfer to the membrane for overnight. The membrane was soaked in 5× SSC for 5 minutes to remove bits of gel or particles from membrane and then dried at room temperature. To immobilize the nucleic acid on the membrane, the blot was baked at 80°C for 1 hour. The membrane was stored in a dry place at room temperature.

2.5.2 Southern hybridization

The Nylon membrane was rolled in the hybridization tube and prehybridized with 10 ml hybridization buffer (50% formamide, 5× SSC, 20 mM Phosphate buffer, 5× Denhardt's solution, 100 μg/ml heat-denatured Herring sperm DNA, 0.5% SDS) at 50^{o}C for 1 hour in hybridization oven with gentle agitation. After prehybridization, the FgLGMN-1 and/or FgLGMN-2 DIG-labeled PCR probes were denatured at 95°C for 5 minutes and added immediately to the buffer (not directly pipette onto the membrane!). The membrane carrying the fragmented genomic DNA was hybridized at 50°C, overnight. To get rid of the unbound and nonspecific binding probes, the membrane was washed with washing buffer 1 (2× SSC, 0.1%SDS) at 55°C for 30 minutes and washing buffer 2 (0.1× SSC, 0.1%SDS) at 55°C for 1 hour under constant agitation. The nylon membrane was taken out from the hybridization tube and used for immunological detection further.

2.5.3 Immunological detection

The membrane was rinsed briefly in maleic washing buffer (0.1 M Maleic acid, 0.15 M NaCl, pH 7.5, 0.3% Tween 20) and incubated in blocking solution (1×) (Roche, Penzberg, Germany) for 30 minutes. After blocking the membrane, the anti-DIG conjugated with alkaline phosphatase was diluted to 1:5,000 in blocking solution (1×) and added to incubate with the membrane for 30 minutes. The membrane was then washed twice with maleic washing buffer for 15 minutes each, and equilibrated in

detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5) for 5 minutes. The membrane was incubated with the NBT/BCIP solution for few minutes to overnight in the dark. When the color development reached the optimal level, the reaction was stopped by washing the membrane several times with DDW. The membrane was then dried and kept for analysis of the result.

2.6 Northern hybridization analysis

Approximately 10-30 μg of total RNA from 2-, 4-, and 6-week old juveniles, and adult *F. gigantica* was heated in 50% formamide/2.2 M formaldehyde in 1× MOPS buffer (20 mM 3-Morpholinopropane-sulfonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.0) at 65°C for 5 minutes and cooled to room temperature. The prepared RNA were mixed with 2× eletrophoretic sample buffer containing 0.5 μg/ml ethidium bromide and loaded to 2.2 M formaldehyde, 1% agarose gel. The gel was run in running buffer (1x MOPS) at 70 V. After that, the formaldehyde agarose gel was rinsed twice in DEPC treated DDW and equilibrated in 20× SSC. The transferring of RNA onto nylon membrane was done as previously described in the Southern transfer protocol (section 2.5.1). The nylon membrane was hybridized with DIG labeled antisense RNA probes of FgLGMN-1 and/or FgLGMN-2 (described in section 3.2) at 60°C, overnight. For the stringent washing step, the membrane was washed with washing buffer 1 at 65°C for 30 minutes and then with washing buffer 2 at 65°C for 1 hour. The hybridized membrane was detected as previously described in immunological detection (section 2.4.1).

2.7 Stage-specific Reverse Transciptase Polymerase Chain Reaction (RT-PCR)

The FgLGMN-1 cDNA was amplified with forward primer (5'-GCATGCATGCACTTCTGTTTGCTG-3') and reverse primer (5'-CTGCAGGATCACTCACAAGCGGTG-3'). The FgLGMN-2 cDNA with forward primer (5'-GGATCCATGTTATCCATGCAAATTTTATTC-3') and reverse primer (5'-CTGCAGTCAAACACAAACATCATG-3') according to "Protocol Using QIAGEN OneStep RT-PCR kit (QIAGEN, Germany).

Total RNA of various developmental stages (metacercariae, 1-, 2-, 4- and 6-week old juveniles and adults) was diluted to 100 pg/µl and mixed with the reaction according to 10 µl of 5× QIAGEN OneStep RT-PCR Buffer, 2 ml of 40 mM dNTP mix, 1 µl forward primer, 1 µl reverse primer, 2 µl QIAGEN OneStep RT-PCR Enzyme Mix and RNase-free H_2O to bring the volume to 50 µl. The reactions were gently mixed and spun down. PCR was performed using the following conditions: 50°C for 30 minutes, 95°C for 15 minutes, 30 cycles of 95°C for 1 minute, 45°C for 1 minute, and 72°C for 1 minute then 72°C for 10 minutes and hold at 10°C. Samples of the PCR products were analyzed on 1% agarose gel. After that, the agarose gel containing PCR products were prepared, transferred to nylon membrane and hybridized with gene-specific PCR probes according to the Southern hybridization technique as previously described (section 2.5).

3. Detection of FgLGMN-1 and FgLGMN-2 transcripts distributed in the tissues of *F. gigantica* at different developmental stages

3.1 Tissue preparation

Whole or dissected adult and juvenile *F. gigantica* were placed in snap-cap glass vials and preserved the tissue preserved with freshly prepared 4% paraformaldehyde in 0.01 M PBS, pH 7.2 (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) at 4°C for 4 hours. After fixation was completed, the fixative was poured off and replaced with 50% ethanol and immediately changed to fresh 50% ethanol. The parasites were incubated for 20 minutes and 50% ethanol changed three times. Then, the tissues were incubated three times in 70% ethanol, 20 minutes each and twice in 80%, 95% and 100% ethanol, respectively for 20 minutes each. The absolute ethanol was replaced with xylene and equilibrated twice for 10 minutes each. Xylene was poured off, 5 ml fresh xylene added to each vial and an equal amount of molten wax was added using a hot glass pipette (transfer quickly to avoid hardening). The sample was mixed and left overnight at room temperature. The samples were transferred to a 60°C oven to melt wax/xylene mixture. The wax/xylene mixture was poured off and immediately fresh molten wax was added to the vial with a hot glass pipette. The vials were put back into the 60°C oven and incubated for 1

hour. The embedding molds were prepared and filled with molten paraffin wax. After that, the tissues were immediately transferred to the wax-filled mold using hot forceps. An embedding ring was placed on the mold, filled with paraffin wax and the embedding ring was labeled to facilitate future identity of samples. Cast blocks were left at room temperature to harden completely and stored in a dry place. The sections were cut at 8 μm thick by a microtome and placed on Histogrip (Zymed, USA) treated slides containing a drop of DEPC-treated H_2O on top. The ribbons of the sections were transferred onto the drop and the slides transferred onto a 45°C heating plate for stretching the sections. After complete stretching, the slides were removed from the heating plate and the DEPC-treated H_2O was carefully removed. Slides were dried at room temperature and stored in a dry place.

3.2 Labeling RNA probes by *in vitro* transcription using DIG RNA Labeling Mix

The pTriplEX2 plasmids containing FgLGMN-1 and -2 fragments were linearized with restriction endonucleases (*Kpn* I or *Xho* I) which created a 5'-overhang at the end of the insert position. Then, the linearized plasmid DNA was run on a 0.7% agarose gel and purified (QIAEX II DNA extraction from agarose gel, QIAGEN, Germany). To synthesize the RNA probe, one microgram of purified, linearized plasmid DNA was mixed in the reaction tube containing 2 μl of 10× DIG RNA labeling mix, 2 μl of 10× transcription buffer, a suitable RNA polymerase (SP6, T7 or T3) and DEPC-treated H_2O to bring the volume up to 20 μl. The reaction mixture was mixed, briefly spun down and incubated for 2 hours at 37°C. Twenty units of DNase I, RNase-free was added to remove the template DNA and incubated for 15 minutes at 37°C. The DIG-labeled RNA transcription probes were precipitated by adding 2.5 μl 4 M LiCl and 75 μl prechilled absolute ethanol and mixed well. The mixture tubes were left for at least 30 minutes at -70°C and then centrifuged at 13,000 ×*g* for 15 minutes at 4°C. The Ethanol was decanted and the pellet washed carefully with 50 μl cold 70% ethanol. The pellet was harvested by centrifugation at 13,000 ×*g* for 5 minutes. After removing the ethanol, the pellet was dried briefly under vacuum and dissolved in 50 μl sterile, RNase free DDW. To protect degradation of RNA by

RNase, one microliter of RNase inhibitor was added and used immediately or stored at -70°C.

3.3 Determination of labeling efficiency

The DIG-labeled RNA probes and labeled control RNA were prepared as a dilution series in RNA Dilution buffer (sterile DEPC treated H_2O: 20× SSC: Formaldehyde in the ratio 5: 3: 2) as described in the table 5

The solution of tubes 3-10 from the DIG-labeled RNA probes and the labeled control RNA were applied in 1 µl spots onto the nylon membrane. The nucleic acids were fixed to the membrane by cross linking with UV-light. The colorimetric detection procedure was done according to Immunological detection protocol, as previously described.

Table 5 Preparation of RNA probe dilution for determination of labeling efficiency

Tube	RNA (µl)	From tube #	RNA Dilution Buffer (µl)	Dilution	Final concentration
1	-	Dilution of probe and control	-	-	10 ng/µl
2	2	1	18	1:10	1 ng/µl
3	2	2	198	1:100	10 pg/µl
4	15	3	35	1:3.3	3 pg/µl
5	5	3	45	1:10	1 pg/µl
6	5	4	45	1:10	0.3 pg/µl
7	5	5	45	1:10	0.1 pg/µl
8	5	6	45	1:10	0.03 pg/µl
9	5	7	45	1:10	0.01 pg/µl
10	0	-	50	-	0

3.4 *In situ* hybridization

3.4.1 *In situ* hybridization

The sections were dewaxed with fresh xylene twice for 10 minutes each and rehydrated with 100% ethanol twice for 5 minutes each. Then, the sections were rehydrated in 70%, 50%, 25% ethanol, respectively for 5 minutes each and twice with DEPC treated water for 5 minutes each. After that, the sections were incubated twice in 1× DEPC-treated PBS for 5 minutes each and with 1× PBST (1× PBS, 0.1% Tween20) twice for 5 minutes each. To deproteinize, the sections were equilibrated with 3 μg/ml of Proteinase K (Fermentas Life Sciences, Vilnius, Lithuania) in 1× PBST for adult *F. gigantica* and 0.03 μg/ml for juvenile at 37°C for 2-3 minutes (add Proteinase K 20 mg/ml to warm Proteinase K buffer 20 minutes before incubation). The reaction was stopped by quick rinsing in 1× PBST, twice in 1× PBST containing 10 mg/ml glycine, 5 minutes each and then twice in 1× PBST, 5 minutes each. The tissues were re-fixed with PBS containing 4% paraformaldehyde at 4°C for 10 minutes and washed again in1× PBST containing 10 mg/ml glycine and then 1× PBST.

The tissue sections were prehybridized in 300 μl hybridization buffer (50% formaldehyde, 5× Denhardt's, 5× SSC, 0.1 mg/ml salmon sperm DNA, 1 mg/ml Heparin, 0.2 mg/ml RNA, 10 mM DTT) for 1 hour at 48°C. After the prehybridization mixture was drained, the 10 ng DIG-labeled LGMN senses- and antisense-RNA probes were added to the hybridization buffer and to denature secondary structures incubated at 70°C for 5 minutes. Each slide was incubated in 30 μl of hybridization buffer at 48^{o}C in humid chamber by covering each slide with cover slip. On the following day, the slides were immersed immediately in prewarmed 4× SSC, 0.1%SDS to remove the coverslips and changed to prewarmed 4× SSC, 0.1%SDS at 48°C for 15 minutes, twice. After that, the sections were washed once at 48°C for 15 minutes with 1× SSC, 0.1% SDS and once at room temperature for 15 minutes. The washing buffer was changed twice with 0.5× SSC, 0.1% SDS at room temperature for 15 minutes and equilibrated in NTE buffer (0.5 M NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0) containing 3 μg/ml RNase A for adult parasite and 0.1 μg/ml for juvenile for 30 minutes at 37°C and washed twice in 0.1× SSC for 15 minutes at room temperature.

3.4.2 Immunological detection

The sections were washed on a shaking platform once with buffer 1 (100 mM Tris-HCl,150 mM NaCl, pH 7.5) for 10 minutes and then incubated with buffer 1 containing 0.05% Tween20 and 2% normal goat serum for 30 minutes at room temperature. To detect transcription products in the tissue, the sections were incubated for 2 hours in a humid chamber with buffer 1 containing 0.05% Tween 20, 1% normal goat serum and 1:5,000 of sheep anti-DIG-alkaline phosphatase. The sections were washed twice in Buffer 1 for 10 minutes each on a shaking platform at room temperature and changed twice with Buffer 2 (100 mM Tris-HCl pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20) for 10 minutes each. The sections were then incubated in 500 µl of the staining solution (NBT/BCIP) containing 1 mM levamisole in a humid chamber for approximately 1-2 hour in the dark. The reaction was stopped by incubating the slides in Buffer 3 (TE buffer; 10 mM Tris-HCl pH 8, 1 mM EDTA) twice for 10 minutes each and washed twice in DEPC-treated water for 5 minutes each. The slides were then mounted with 90% glycerol in buffer 3 and analyzed under the microscope.

4. Expression, purification of recombinant FgLGMN-1 and FgLGMN-2 proteins and antibody production

4.1 Production of recombinant FgLGMN-1 and FgLGMN-2 genes

PCR primers were designed to amplify the coding sequences of the *F. gigantica* LGMN-1 and -2 genes encoding the putative mature proteins without the regions encoding the signal peptides. FgLGMN-1 was amplified with forward primer (5'-AAGCTTGAAGGCGGTGGGGGAAAG-3') containing a *Hin*d III site (5'-CTCGAGGATCACTCACAAGCGGTG-3') and reverse primer containing *Xho* I site. FgLGMN-2 with forward primer containing a *Hin*d III site (5'-AAGCTTAAGACCGGAAAGAATTGGGC-3') and reverse primer containing an *Xho* I site (CTCGAGTCAAACACAAACATCATGAATCAC-3'); the restriction endonuclease sites are underlined in the primer sequence.

The following components were combined in the reaction tube, 1 µl of 100 pg/µl recombinant pTriplEx2 plasmid containing LGMN DNA fragments, 37.5 µl of DDW, 5 µl of 10× PCR buffer, 1 µl of 50× dNTPs mix, 1 µl of forward primer, 1 µl of reverse primer, and 0.5 µl of Taq DNA polymerase enzyme (5 U/µl) (Fermentas Life Sciences, Vilnius, Lithuania). The reactions were gently mixed and spun down. PCR was performed using the following conditions: 94°C for 5 minutes, 30 cycles of 94°C for 1 minute, 45°C for 1 minute, and 72°C for 1 minute then 72°C for 8 minutes and hold at 10°C. Samples of the PCR products were analyzed on a 1% agarose gel. The DNA bands were excised from the agarose gel following the QIAEX II DNA gel extraction kit (QIAGEN, Germany).

4.2 Cloning of FgLGMN-1 and FgLGMN-2 into an expression vector and transformation of *E. coli* with recombinant plasmids.

4.2.1 Ligation of FgLGMN-1 and FgLGMN-2 to pGEM®-T Easy vector

The PCR products were cloned into the pGEM®-T Easy vector by adding the following components in a 1.5 ml reaction tube, 2 µl DNA, 2 µl of vector (500 ng/ul), 1 µl of 10× ligation buffer, 1 µl of ATP (10 mM), 1 µl of T4 DNA Ligase, and 3 µl of DDW. The reaction sample was mixed, briefly spun down and incubated at 4°C overnight. The ligation products were introduced into *E. coli* XL1-blue competent cell by transformation. The transformant *E. coli* XL1-blue were spread on LB agar plates containing 100 µg/ml ampicillin, 0.5 mM IPTG and 80 µg/ml X-Gal and incubated at 37°C overnight. Several white colonies were selected and positive clones screened by colony-PCR technique (described in section 4.2.2). The positive clones were subjected to purify the recombinant plasmid by plasmid mini-preparation kit (QIAGEN, Germany) as described before and digested with restriction endonucleases (*Hin*d III and *Xho* I) to screen for the presence of inserts of the correctly size.

4.2.2 Colony- Polymerase Chain Reaction technique

Each white colony was picked and resuspended in100 µl of sterile DDW. The DNA fragments specific to FgLGMN-1 and FgLGMN-2 were amplified by using gene specific primers. The FgLGMN-1 and -2 were amplified with forward primer

and reverse primer as described in section 4.1. The following components were combined in the reaction tube, 1 µl of colony suspension (1 colony was resuspend in 100 µl of sterile DDW), 37.5 µl of DDW, 5 µl of 10× PCR buffer, 1 µl of 50× dNTPs mix, 1 µl of forward primer, 1 µl of reverse primer, and 0.5 µl of Taq DNA polymerase enzyme (5 U/µl). The reactions were gently mixed and spun down. PCR was performed using the following conditions: 94°C for 5 minutes, 30 cycles of 94°C for 1 minute, 45°C for 1 minute, and 72°C for 1 minute then 72°C for 10 minutes and hold at 10°C. Samples of the PCR products were analyzed on a 1% agarose gel and detected under UV light. From positive clones the recombinant plasmids were isolated by plasmid mini-preparation kit (QIAGEN, Germany, described below).

4.2.3 Digestion reaction of recombinant pGEM®-T Easy containing FgLGMN-1 and FgLGMN-2

The FgLGMN-1 and -2 DNA fragments were released from pGEM®-T Easy by digestion with the *Hin*d III and *Xho* I restriction endonucleases. The restriction recognition sites have been introduced by the PCR primers. The reaction sample contained 5 µl pGEM®-T Easy plasmid containing FgLGMN-1 or FgLGMN-2, 2 µl 10× buffer Y+ reaction buffer, 0.5 µl *Hin*d III (10 U/µl, Fermentas Life Sciences, Vilnius, Lithuania), 0.5 µl *Xho* I (10 U/µl, Fermentas Life Sciences, Vilnius, Lithuania) and 12 µl sterile DDW. The reaction mixtures were completely mixed, spun down, and incubated at 37°C for 2 hours. The digestion products were analyzed in 1% agarose gel electrophoresis. pGEM®-T Easy plasmids containing the correct size of FgLGMN were sent for sequencing (MWG AG Biotech, Germany) to confirm the correct sequence.

4.2.4 Subcloning of FgLGMN-1 and FgLGMN-2 into the prokaryote expression vector pET20b(+)

The FgLGMN-1 and -2 DNA fragments were released from pGEM®-T Easy by digestion with *Hin*d III and *Xho* I restriction endonucleases. The digestion reaction with the restriction endonucleases was performed as described before. The digestion was analyzed by 1% agarose gel electrophoresis. A 1 kb DNA size marker (Fermantas

Life Sciences, Vilnius, Lithuania) was used to estimate size and amount of the digested DNA. The FgLGMN-1 and -2 DNA fragments were purified from the gel with a gel extraction kit (QIAGEN, Germany). The expression vector pET20b(+) (Novagen, UK) was cut with *Hin*d III and *Xho* I and the FgLGMN DNA fragments were inserted using T4 DNA ligase (Promega, USA). The ligation buffer was composed of 100 ng of pET20b(+) vector, 162 ng of DNA fragments, 1 µl Ligase 10× buffer, 1 µl T4 DNA ligase (1 Weiss unit) and nuclease-free H_2O to 10 µl. Appropriated proportion between vector and DNA fragments was calculated by using the formula according to;

$$\frac{\text{ng of vector} \times \text{kb size of insert} \times \text{molar ratio of insert}}{\text{kb size of vector}} = \text{ng of insert vector}$$

* The desired vector: insert ratio was 5:1

The ligation mixture was incubated at 4^{o}C overnight and transformed to *E. coli* XL 1-Blue.

The transformation was done as described before and transformed *E. coli* cells were spread on LB agar plate containing 100 µg/ml ampicillin, 0.5 mM IPTG and 80 µg/ml X-Gal. The plates were incubated at 37°C overnight. Several single isolated white colonies were subjected for isolation of plasmid DNA and tested for the size of the inserted fragments with *Hind* III and *Xho* I restriction enzymes. pET20(+) plasmid containing the correct size insert were used for transformation of *E. coli* BL21(DE3)pLysS (Novagen, UK) by electroporation.

4.2.5 Preparation and storage of electrocompetent *E. coli* BL21(DE3)pLysS

The glycerol stock of *E. coli* BL21(DE3)pLysS was plated onto LB agar containing 34 µg/ml chloramphenicol. A single isolated colony was inoculated in 2 ml LB broth containing 34 µg/ml chloramphenicol and the starter culture was grown overnight in 37°C shaker at 250 rpm. On the following day, the starter was inoculated into 200 ml of LB broth containing 34 µg/ml chloramphenicol. The bacterial cells

were grown until OD_{600} equal to 0.6, the cells removed from the shaker and placed on ice. Bacteria were harvested by centrifugation at 4,000 ×*g* for 30 minutes at 4°C. The centrifuge bottle was placed on ice, the supernatant was removed immediately and the pellet was gently resuspended in 200 ml ice-cold DDW. These steps were performed twice. After that, the bacteria were harvested by centrifugation at 4,000 ×*g* for 30 minutes at 4°C and resuspended in 20 ml ice-cold 10% glycerol DDW. These steps were also done twice. The supernatant was removed and the cell pellet resuspended in 2 ml of ice-cold 10% glycerol DDW and aliquots of 100 µl of suspension were prepared in 1.5 ml microcentrifuge tubes on ice. The competent cells were fast frozen in liquid N_2 and stored f at -70°C.

4.2.6 Electroporation of electrocompetent *E. coli* BL21(DE3)pLysS

The frozen electrocompetent *E. coli* BL21(DE3)pLysS aliquots and pET20(+) plasmid containing FgLGMN DNA fragments were thawed on ice. Three microliters of plasmid DNA were mixed with 100 µl of electrocompetent cells and placed into a pre-cooled 1 mm electrocuvette (Eppendorf, Germany). The cuvette was dried off any moisture from outside and immediately placed into the plastic holder. The holder was slid into position and cells were zapped. The condition for transformation was set according to 25 µFD, 200 Ω, and 2.5 kV for 4 msec. 900 µl of SOC medium was immediately added to cells and transferred to a 1.5 ml microcentrifuge tube. The Transformed bacteria were outgrown for 1 hour at 37°C in a shaking incubator at 250 rpm and plated onto LB agar containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. Plates were incubated at 37°C overnight. Several colonies were picked and the positive clones tested by colony-PCR that was described before.

4.3 Expression and purification of recombinant FgLGMN-1 and FgLGMN-2 proteins

4.3.1 Screening of small-scale expression cultures

Five positive colonies were cultured in 1.5 ml LB medium containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol and grown overnight. On the following day, 10 ml of prewarmed LB medium containing 100 µg/ml ampicillin and

34 μg/ml chloramphenicol were inoculated with 500 μl of the overnight culture and cultured at 37°C with vigorous shaking (250 rpm), until OD_{600} = 0.5-0.7. One milliliter of culture medium was collected in 1.5 ml microcentrifuge tube as a non-induced control. To induce the expression of recombinant protein, IPTG was added to the remaining culture medium to a final concentration of 1 mM and grown for additional 4 hours. One milliliter of culture medium was collected into a 1.5 ml microcentrifuge tube. Both non-induced and induced bacterial cells were harvested by centrifugation for 1 minute at 12,000 ×*g*, and the supernatants were discarded. Pellets were resuspended in 50 μl 2× reducing sample buffer and incubated 20 minutes at room temperature. To eliminate cell debris, the suspension was centrifuged at 12,000 ×*g* for 10 minutes at room temperature and the expression of protein was analyzed by SDS-PAGE (described in section 4.4).

4.3.2 Time-course analysis of protein expression

The clone which showed strong expression was subjected to analyze the time-course of protein expression. A single colony was inoculated in 5 ml of LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol and cultured overnight. 2.5 ml of the overnight culture were inoculated into 50 ml LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol and cultured at 37°C with vigorous shaking (250 rpm), until OD_{600} = 0.5-0.7. 5 ml of the bacterial suspension were collected as a control and then the expression of protein was induced by adding 1 mM IPTG. After that, five milliliters of the culture fluid were collected at 1, 2, 3, and 4 hour(s). All samples were centrifuged at 4,000 ×*g* for 20 minutes and the supernatant was discarded. The pellets were resuspended in 1 ml 2× reducing sample buffer and incubated 20 minutes at room temperature. To eliminate cell debris, the suspension was centrifuged at 12,000 ×*g* for 10 minutes at room temperature and then the expression of protein was analyzed by SDS-PAGE (described in section 4.4).

4.3.3 Determination of target protein solubility

The *E. coli* BL21(DE3)pLysS containing the recombinant pET20b(+)-FgLGMNs were inoculated in 10 ml LB medium containing 100 μg/ml ampicillin and

34 μg/ml chloramphenicol in a 50 ml flask and grown overnight at 37°C with shaking at 250 rpm. On the following day, 2.5 ml of the overnight culture were inoculated into 50 ml of prewarmed media (with antibiotics). The bacteria were grown at 37°C, with vigorous shaking (~300 rpm), until the OD_{600} was 0.5-0.7. A sample of 5 ml was taken immediately before induction (non-induced control). The cells were harvested by centrifugation at 4,000 ×*g* for 20 minutes at 4°C and resuspended in 100 μl 2× SDS-PAGE sample buffer. The sample was frozen at -20°C until needed for SDS-PAGE. To induce expression of protein, 1 mM IPTG was added to the cultures and grown for an additional 4 hours and 5 ml sample was collected and centrifuged at 4,000 ×*g* for 20 minutes and 4°C. The cell pellet was resuspended in 5 ml native lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). The bacterial cells were lysed by sonication 6 × 9 sec with 9 seconds pauses at 30% amplitude (Sonic model VC750, Connecticut, USA). The lysate was kept on ice at all times. The lysate was centrifuged at 12,000 ×*g* at 4°C for 30 minutes and the supernatant was collected (crude extract A, soluble protein) and saved on ice. The pellet was resuspeneded in 5 ml lysis buffer. This is a suspension of the insoluble matter (crude extract B, insoluble protein). The crude extracts were analyzed by SDS-PAGE (as described in section 4.4).

4.3.4 Expression of recombinant FgLGMN-1 and FgLGMN-2proteins

The starter was prepared by inoculation of a well-single isolated colony of the *E. coli* BL21(DE3)pLysS containing recombinant pET20b(+)-FgLGMNs into 20 ml LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol and cultured at 37°C overnight with vigorous shaking (250 rpm). 20 ml of overnight culture was inoculated in a liter LB (with antibiotic) and grown at 37°C with vigorous shaking until the OD_{600} was 0.5. A 5 ml sample was taken immediately before induction (non-induced control). The cells were harvested by centrifugation at 4,000 ×*g* for 20 minutes and 4°C and resuspended in 100 μl 2× sample buffer. The sample was frozen at -20°C until needed for SDS-PAGE. The expression of protein was induced by adding IPTG to a final concentration of 1 mM to the cultures and grown for additional 4-5 hours and a 5 ml sample was collected (induced control) and centrifuged at 4,000 ×*g* for 20

minutes and 4°C. The cell pellet was resuspended in 100 µl 2× sample buffer and kept at -20°C until use.

4.3.5 Purification of recombinant FgLGMN-1 and FgLGMN-2 proteins

The frozen bacteria pellets were thawed on ice for 15 minutes and resuspended in buffer B (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M Urea, pH 8.0) at 5 ml per gram wet weight. The cells were stirred for 30 minutes at room temperature and then sonicated 6 × 9 seconds with 9 seconds pauses at 30% amplitude (Sonic model VC750, Connecticut, USA). The lysate was centrifuged at 12,000 ×*g* for 30 minutes at room temperature and the supernatant was collected in a new sterile 15 ml conical tube. One milliliter of 50% Ni-NTA slurry (Qiagen, Germany) was added to 4 ml lysate (~5 mg of 6× His-tagged protein) and mixed gently by shaking at 200 rpm on a rotary shaker for 1.30 hours at room temperature. The lysate-resin mixture was loaded carefully into an empty column with the bottom cap still attached. The bottom cap was removed and the flow-through was collected. The column was washed twice with 4 ml buffer C (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M Urea, pH 6.3). The recombinant protein was eluted 4 times with 0.5 ml buffer D (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M Urea, pH 5.9), followed by 4 times with 0.5 ml buffer E (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M Urea, pH 4.5). All fractions were collected and analyzed by SDS-PAGE (as decribed in section 4.4).

4.3.6 Dialysis of elution fractions

To eliminate urea, all elution fractions were pooled together and dialyzed against 0.01 M PBS, pH 7.4 at 4°C for 48 hours. The recombinant proteins were precipitated after complete dialysis. The dialyzed recombinant proteins were obtained by centrifugation at 12,000 ×*g* for 20 minutes at 4°C. The pellets were kept at -20°C for further analysis.

4.4 Analysis of recombinant proteins by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

4.4.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis in the presence of 1% (w/v) sodium dodecyl sulfate (SDS-PAGE) was used to analyze the complexity of protein profiles, sensitivity and resolution of the antigens prior to electroblotting onto nitrocellulose paper for Western blot analysis. The techniques described by Laemmli (1970) were followed with some modifications. The 10×10 cm vertical slab gel was prepared by using the casting apparatus (Hoefer, Germany) while electrophoresis was done in an electrophoretic chamber (Hoefer, Germany) and with an electric power supply (Amersham Biosciences model EPS 301, USA). A 4% acrylamide stacking gel and 12% acrylamide separating gel were used in this process (Appendix A, section 11.7 and 11.8).

Samples were denatured by diluting with equal volumes of 2× sample buffer (Appendix A, section 11.1) and heating at 100°C for 4 minutes before carefully loaded into the slots in the stacking gel. The desired amount of the sample was carefully applied into each well. Broad range molecular weight standard (Bio-Rad, cat no. 161-0317, U.S.A.) was included in one slot of each gel slab. Care was taken not to contaminate the adjacent wells with the samples.

Electrophoresis was carried out in an electrophoretic chamber at a constant current (40 mA). Electrophoresis was stopped when the dye marker reached about 0.5 cm from the bottom edge of the gel. The total running time was about 45 minutes.

After electrophoresis, the gel was removed from the glass. It was then either stained and destained or proceeded to the transblotting onto the nitrocellulose membrane for Western blot analysis.

4.4.2 Staining and destaining of protein bands

Protein bands were revealed by soaking the gel in Coomassie brilliant blue staining solution and destaining solution (Appendix A, section 12) as follows:

The slab gel from SDS-PAGE containing separated proteins was fixed in the fixing solution at room temperature for 1 hour. After removing the fixing solution, gels were placed in a colloidal Coomassie brilliant blue G250 staining solution at room temperature for overnight on an orbital shaker or platform rocker. After that, the stain was removed and the gel was neutralized in a neutralization solution. Gels were changed to washing solution few minutes or until the background level was adequately reduced. For stabilization of color, gels were equilibrated in stabilizing solution. When destaining was completed, the gel was dried on a paper (Whatman no. 3 and/or cellophane membrane backing, Bio-Rad, U.S.A.) using a gel dryer (Model 583 gel dryer, Bio-Rad, USA).

4.5 Production of polyclonal antibodies against the recombinant FgLGMN-1 and FgLGMN-2 proteins

4.5.1 Experimental animals

Female New Zealand white rabbits were purchased from the center of experimental animals, Mahidol University, Nakorn Pathom, Thailand. The animals were 7-8 weeks old, weighing about 1.2 kg at the beginning of the experiment. The rabbits were immunized with recombinant FgLGMN-1 and FgLGMN-2 proteins. The immunized sera were subjected to characterize proteins. Non-immunized serum was also prepared and used as negative control serum. The sera were used in either Western blot analysis and immunohistochemical staining.

4.5.2 Immunization of the New Zealand white rabbit

To produce antibodies against recombinant protein, the recombinant protein pellet was dissolved in 2× sample buffer and size-separated in 12% SDS-PAGE. To indicate the correct size, the polyacrylamide gel was stained with Coomassie G-250 staining solution (described in section 4.4.2) for 2 h and the gel was washed with 0.01 M PBS, pH 7.4. A band of the gel was cut out at the expected size of the recombinant protein and ground in a mortar. Small pieces of the gel were passed through several times with the 22 G needle for injection into the experimental animal. The New Zealand White rabbit was immunized with gel embedded protein following the

immunization protocol. The pre-immune serum was collected before immunization. The rabbit was primary injected intramuscular and then immunized again at 2 weeks interval (1st Booster and 2nd Booster) at the same route. Two weeks after 2nd Booster, blood was collected from the ear artery and incubated overnight at 4°C. Serum was obtained by centrifugation at 4,000 ×*g* for 10 minutes at room temperature. The serum was aliquoted and kept in -20°C for further immunological studies.

5. Characterization of FgLGMN-1 and FgLGMN-2 proteins

5.1 Preparation of crude *F. gigantica* extracts (CWAg)

Frozen adult *F. gigantica* were thawed and ground up as one gram per two milliliter of homogenization buffer: 0.01 M PBS, pH 7.2 containing 1% Triton X-114, 2 mM PMSF, 5 mM Iodoacetamide, and 1 mM EDTA, by using a tissue homogenizer (Ika,Germany or glass tissue grinder). The homogenates were then sonicated at 30% amplitude at 5 × 9 seconds pulse on and 9 seconds pulse off. The suspensions were centrifuged 12,000 ×*g* for 30 minutes at 4°C and the supernatants were collected. The CWAg was aliquoted 200 µl into microcentrifuge tubes. Concentration of proteins was measured and the proteins were stored at -70°C for further study.

5.2 Preparation of excretory-secretory products (ESAg) from *F. gigantica*

Adult *F. gigantica* were collected from the liver, bile duct and gall bladder of the naturally infected cattle killed at a local abattoir in Pathumthani province. The parasites were washed several times in 0.01 M PBS, pH 7.2 incubated an hour in each period at room temperature. The clean parasites were cultured in 0.01 M PBS, pH 7.2 and incubated in 5% CO_2, 10% O_2 at 37°C for 4 hours. The supernatant was collected and then centrifuged 4,000 ×*g* at 4°C for 20 minutes. The supernatant was concentrated by using the spin column concentrator (Microcon YM-3, Millipore, USA) and stored at -70°C.

5.3 Measurement of protein concentration

The concentration of proteins was measured by Bradford's method using a protein assay kit (Bio-Rad, USA). Firstly, BSA was prepared as standard proteins

desired in different concentration; 0.1, 0.2, 0.3, 0.4, 0.5 mg/ml. 10 µl of standard proteins and appropriate diluted protein samples were mixed with 200 ml of Bradford reagent and gently mixed. The reaction was performed in a 96-well microtiterplate. The reactions were incubated at room temperature for at least 5 minutes but not more excess than 1 hour. The visible color was measured at 595 nm by ELISA reader spectrophotometer (Anthos model 2020, Austria). A standard curve was created from the OD values of standard proteins (Y-axis) and standard protein concentration (X-axis). The concentration of protein samples was measured by comparing to a standard curve.

5.4 Analysis and characterization of recombinant proteins and native proteins

5.4.1 Western blot analysis

The recombinant proteins (500 ng), ESAg (10 µg) and CWAg (10 µg) were size-separated in 12% SDS-PAGE (described in section 4.4). Then, the proteins in the gel were electro-transferred to PVDF membrane (Hybond P, Amersham Biosciences, USA) by using a semi-dry electrophoretic transfer machine (Multiphor II Nova Blot Unit, Pharmacia LKB Biotechnology, Sweden) followed by semi-dry blotting technique. Firstly, the PVDF membrane was soaked in absolute methanol and then equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% [v/v] methanol). The protein transfer was performed at 70 mA for 80 minutes. The transferred proteins were stained with Ponceau S solution (0.25% [w/v] Ponceau S, 1% [v/v] acetic acid) by briefly soaking the membrane and then washing with DDW until the background became clear.

5.4.2 Immunoblot analysis

The blotted membranes were blocked non-specifically with blocking solution (3% BSA, 0.2% gelatin in 0.01 M PBS, pH7.4) for 1 hour, at room temperature. The membranes were washed thoroughly with washing buffer (0.01 M PBS pH 7.4, 0.05%Tween 20). Rabbit anti-rFgLGMN-1 or -2 were diluted in diluent (0.2% BSA, 0.2% gelatin in 0.01 M PBS, pH 7.4) to 1:800 and incubated with the membranes for 1 hour, at room temperature. To eliminate unbound antibodies, the

membranes were washed with washing buffer three times, 5 minutes each. The goat anti-rabbit Ig conjugated with alkaline phosphatase (Dako, Denmark) was diluted to 1:2,000 and incubated with the membranes. The excess antibodies were washed out with washing buffer three times, 5 minutes each. For colorimetric detection, membranes were firstly equilibrated in substrate buffer (0.15 M Tris-HCl, pH 9.5) for 5 minutes, at room temperature and were then changed to NBT/BCIP solution (KPL, USA). The reactions were stopped when visible bands appeared by washing several times with DDW.

5.5 Analysis of immune response from *F. gigantica* infected mice against recombinant proteins

500 ng of each recombinant proteins (rFgLGMN-1 and -2) were size-separated in 12% SDS-PAGE as previously described. Then, the proteins were transferred to PVDF membrane by using an electrophoretic-transfer-machine. The blotted membranes were blocked non-specifically with blocking solution (3% BSA, 0.2% gelatin in 0.01 M PBS, pH7.4) for 1 hour, at room temperature. The membranes were washed thoroughly with washing buffer (0.01 M PBS pH 7.4, 0.05%Tween 20). Sera from infected mice at 0, 1, 2, 4 and 6 week(s) were diluted in diluent (0.2% BSA, 0.2% gelatin in 0.01 M PBS, pH 7.4) to the desired dilution and incubated with membranes for 1 hour, at room temperature. To eliminate unbound antibodies, the membranes were washed with washing buffer three times, 5 minutes each. The rabbit anti-mouse Ig conjugated with alkaline phosphatase (Dako, Denmark) was diluted to 1:2,000 and incubated with the membranes. The excess antibodies were washed out with washing buffer three times, 5 minutes each. For colorimetric detection, membranes were firstly equilibrated in substrate buffer (0.15 M Tris-HCl, pH 9.5) for 5 minutes, at room temperature and were then changed to NBT/BCIP solution (KPL, USA). The reactions were stopped when visible bands appeared by washing several times with DDW.

5.6 Deglycosylation of native legumains with N-Glycosidase F

20 μg of CWA was denatured in 0.5% SDS, 0.04 M DTT at 100°C for 10 minutes. *N*-Glycosidase F (New England Biolabs® Inc., USA) was then introduced to

the mixture at a final concentration of 5 U in the condition presenting 0.05 M Sodium phosphate, pH 7.5 and 1% NP-40. The enzymatic reaction was performed at 37°C for 1 hour. Deglycosylated legumains were analyzed by SDS-PAGE and Western analysis with rabbit-anti recombinant FgLGMNs as previously described.

6. Immunolocalization of native proteins in tissue of 4-week old juvenile and adult parasites by using rabbit anti-rFgLGMNs polyclonal sera

6.1 Removal of paraffin and rehydration

4-week old juvenile and adult *F. gigantica* were prepared as described in section 3.1. Paraffin embedded sections (8 μm) of 4 week-old juveniles and adults were then deparaffined twice with xylene for 5 min each. The tissue sections were rehydrated in serials alcohol dilution with two changes of fresh absolute ethanol for 3 minutes each, 95%, 80% and 70% ethanol for 3 minutes each. The slides were rinsed in gently running tap water for 30 seconds (avoid a direct jet which may wash off or loosen the section).

6.2 Antigen retrieval-unmasking of antigen

The antigenic epitopes were disclosed by antigenic retrieval microwave technique. The slides were washed briefly with microwave antigen retrieval solution (10 mM sodium citrate buffer pH 6.0) and placed in a microwave-resistance container containing antigen retrieval solution. The microwave oven was operated on high power (~700 watts) three times for 5 minutes each. The buffer was cooled slowly at room temperature for at least 20 minutes before proceeding to the next step.

6.3 Inactivation of endogenous peroxidase

Slides were placed on a flat level surface and enough amount of 3% H_2O_2 diluted in DDW was added to cover the whole sections and incubated 30 minutes at room temperature. The tissue section slides were rinsed in 1× PBST (0.01 M PBS pH7.4, 0.1% Tween 20) and washed again in 1× PBST for 2 minutes.

6.4 Primary antibody reaction

Slides were drained, excess fluid was shaken off with a brisk motion and each slide carefully wiped around the sections. The non-specifics were blocked by incubating the sections in blocking solution (10% normal goat serum in 0.01 M PBS pH 7.4) for 20 minutes at room temperature. The blocking solution was removed and the sections incubated in 100 µl primary antibody (immunized or normal) diluted in diluent (1% normal goat serum in 0.01 M PBS pH 7.4) to optimal dilution. The tissue sections were incubated at 4°C overnight in a humid chamber. Unbound antibodies were washed with 1× PBST twice for 5 minutes each with gently shaking.

6.5 Secondary antibody reaction

Anti-rabbit Ig biotinylated secondary antibody (Dako, Denmark) was diluted in diluent to a final dilution of 1:2,000 and then 100 µl applied to cover tissue sections. The slides were incubated in a humid chamber for at least 30 minutes at room temperature. The slides were washed with 1× PBST twice for 5 minutes each with gentle shaking. Tissue sections were incubated in 0.05 M TBS, pH 7.6 for 2 minutes. The ExtrAvidin peroxidase was prepared following the instruction protocol (ABComplex/HRP, DakoCytomation, Denmark). The appropriate amount was applied to cover the tissue sections and incubated in a humid chamber for 30 minutes at room temperature. The slides were washed in 1× PBST twice for 5 minutes each with gentle shaking.

6.6 Color development

The freshly prepared AEC (Aminoethyl carbazole) substrate kit (Zymed Laboratories Inc., Germany) was applied to cover the tissue section and incubated at room temperature in the dark. Until the staining reaches the optimal level, the color development was stopped by rinsing in 2-3 changes of DDW. The tissue sections were mounted with 90% glycerol and subjected to microscopic examination.

7. Autocatalytic processing and functional assay of recombinant FgLGMN and native proteins

7.1 Refolding of recombinant FgLGMN proteins

Purified recombinant FgLGMN-1 and -2 were refolded by step-wise dialysis. In detail, dialysis tubes containing recombinant proteins were equilibrated in 200 ml isotonic buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M Urea, pH 6.3). Refolding was performed by dropping 0.01 M PBS, pH 7.2 to the isotonic buffer until presenting 2 M Urea (600 ml), the flow rate was adjusted to 0.5 ml/min by peristaltic pump (Biorad, model EP-1, USA). Refolded recombinant proteins were obtained and glycerol was added to 30% (w/w). Recombinant proteins were stored at -20°C for further analysis.

7.2 Autocatalytic processing of recombinant FgLGMN proteins

10 μg refolded recombinant proteins were incubated with 0.2 M sodium citrate buffer containing 1 mM DTT in different pH (pH 4, 5, 6, 7) at 37°C for 2 hours. Proteins were analyzed by 12% SDS-PAGE and electrophoretically transferred to PVDF membrane. The membranes were incubated with rabbit anti-FgLGMN-1 and -2 polyclonal sera (1:800). Goat anti-rabbit Ig conjugated with alkaline phosphatase (Dako, Denmark) (1:2,000) was added and colorimetric detection was developed by the chromogenic NBT/BCIP substrate.

7.3 Hydrolysis of a synthetic peptide substrate

Approximately 10 μg of autocatalytic recombinant proteins incubated at different pH (pH 7, 6, 5 and 4) were mixed with assay buffer (50 mM sodium acetate, pH 5.4, 1 mM DTT) to a final volume of 197 μl and then 3 μl of synthetic peptide substrate Bz-Asn-pNa (stock solution was prepared at concentration of 15 mg/ml in DMSO) (BACHEM, Switzerland) were added. The reaction mixture was incubated at 37°C for 1 hour, centrifuged at 16,000 ×*g* for 3 minutes and the supernatant transferred to a 96-well microtiter plate. The released pNA was measured at 405 nm by ELISA reader (Anthos model 2020, Austria). The non-incubated reaction mixtures were used as a

blank. The recombinant proteins were boiled at 100°C for 5 minutes and performed in parallel as negative controls. Tests were done in triplicate.

7.4 Legumain activity assay of native parasite extracts

Crude worm extracts (CWAg) and excretory-secretory products (ESAg) were freshly prepared as previously described (section 5.1 and 5.2) and used for analysis of legumain activity. 90 μg of CWAg and ESAg were mixed with assay buffer (50 mM sodium acetate, pH 5.4, 1 mM DTT) to a final volume of 197 μl. Then, 3 μl of 15 mg/ml Bz-Asn-pNA substrate (BACHEM, Switzerland) were added and incubated at 37°C for 1 hour. The mixture was centrifuged at 16,000 ×*g* for 3 minutes and then the supernatant transferred to 96-well microtiterplate. The released pNA was measured at 405 nm by an ELISA reader (Anthos model 2020, Austria). The non-incubated reaction mixtures were used as blanks. The CWAg and ESAg were boiled at 100°C for 5 minutes and performed in parallel as negative controls. Tests were done in triplicate and statistical analysis between samples and negative groups was done by the Mann-Whitney test at 95% confidential limit.

7.5 Legumain inhibitor sensitivity assays

The legumain inhibitor sensitivity assays were performed with crude worm extracts freshly prepared from adult *F. gigantica*. The inhibitors that were used in these assays were cysteine protease inhibitor: Iodoacetamide (IAA), serine protease inhibitor: Phenylmethyl Sulphoxamide (PMSF), metalloprotease inhibitor: chelating agent EDTA.

Approximately 90 μg of crude worm extract was incubated with inhibitor at the final reaction concentrations: IAA (5 mM), PMSF (2 mM), EDTA (1 mM), and cocktail (5 mM IAA, 2 mM PMSF and 1 mM EDTA). Assay buffer (50 mM sodium acetate, 1 mM DTT) was added to bring the final volume to 197 μl. 3 μl of 15 mg/ml Bz-Asn-pNA were added to each reaction tube and the samples were incubated at 37°C for 1 hour. The mixture was centrifuged at 16,000 ×*g* for 3 minutes and the supernatant transferred to a 96-well microtiterplate. The released pNA was measured at 405 nm by an ELISA reader (Anthos model 2020, Austria). The non-incubated reaction mixtures were used as blanks and tests were done in triplicate.