

Cloning and expression analysis of a long type peptidoglycan recognition protein (PGRP-L) from *Xenopus tropicalis*

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Abstract: Peptidoglycan recognition proteins (PGRPs) are a family of pattern recognition receptors (PRRs) of the immune system, which bind and hydrolyze bacterial peptidoglycan. Here, a long type PGRP (PGRP-L) was first cloned in the lower vertebrate species *Xenopus tropicalis* (Xt). The XtPGRP-L possessed a conserved genomic structure with five exons and four introns. The alignment and phylogenetic analysis indicated that XtPGRP-L might be a type of amidase-like PGRP. The 3-D model showed that XtPGRP-L possessed a conserved structure compared with the *Drosophila* PGRP-Lb. During embryonic development, XtPGRP-L was not expressed until the 72 h tadpole stage. In adult tissues, it was strongly expressed in the liver, lung, intestine, and stomach. Furthermore, after LPS stimulation, the expression of XtPGRP-L was up-regulated significantly in the liver, intestine and spleen, indicating that XtPGRP-L may play an important role in the innate immunity of *Xenopus tropicalis*.

Key words: Peptidoglycan recognition protein; Gene clone; Expression analysis; *Xenopus tropicalis*

非洲爪蟾中一种长型肽聚糖识别蛋白的克隆与表达分析

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摘要: 肽聚糖识别蛋白(peptidoglycan recognition proteins, PGRPs)是固有免疫系统中一类重要的模式识别受体。该文首次从两栖类模式生物-非洲爪蟾 (*Xenopus tropicalis*)中克隆得到了一个长型 PGRP(XtPGRP-L)基因。XtPGRP-L 具有 5 个外显子和 4 个内含子的基因组结构, 该结构在进化的过程中比较保守。序列比对与系统进化分析显示 XtPGRP-L 具有保守的酰胺酶活性位点。蛋白质建模显示 XtPGRP-L 拥有保守的 3-D 结构。实时定量 PCR 检测显示, XtPGRP-L 在非洲爪蟾胚胎早期不表达, 到 72 h 蝌蚪期开始表达。在成体的肝脏、肺、肠和胃高表达。同时, 在 LPS 刺激后, XtPGRP-L 在肝脏、肠和胃中呈明显上调表达。结果表明, XtPGRP-L 在非洲爪蟾固有免疫系统中可能具有重要的作用。

关键词: 肽聚糖识别蛋白; 基因克隆; 表达分析; 非洲爪蟾

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Peptidoglycan recognition proteins (PGRPs) are a group of receptors that recognize conserved pathogen-associated molecular patterns (PAMPs) to initiate host immune responses. They were first identified in the

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hemolymph and cuticle of the silkworm (*Bombyx mori*), and were shown to bind peptidoglycan (PGN) and activate the prophenoloxidase cascade (Yoshida et al, 1996). Members of the PGRP family have been characterized in insects, teleosts, and mammals and are evolutionarily conserved. Twelve PGRP genes have been reported in *Drosophila melanogaster*, which are classified into two classes based on length: the short class (PGRP-S) including PGRP-SA, SB1, SB2, SC1A, SC1B, SC2, and SD; and the long class (PGRP-L) including PGRP-LA, LB, LC, LD, and LE. Insect PGRPs have diverse functions and roles such as cell activation and phagocytosis (Werner et al, 2000). In teleost, multiple PGRP genes have been discovered (Chang et al, 2007), some of which exhibit peptidoglycan-lytic amidase activity and broad-spectrum bactericidal activity (Li et al, 2007), and are involved in various pathways (Chang & Nie, 2008; Chang et al, 2009; Wang & Lai, 2010). Unlike insects and fish, only four PGRPs have been identified in mammals (Liu et al, 2000, 2001): PGRP-S (short), PGRP-IA (I for intermediate), PGRP-IB (intermediate), and PGRP-L (long), which also have amidase and antibacterial activities (Li et al, 2007). However, PGRPs have not been reported in other groups of vertebrates.

Using the available genome database of the clawed frog (*Xenopus tropicalis*), it is possible to identify PGRPs from this amphibian model. In this study, a single long type PGRP was first identified from *X. tropicalis*, with its genomic structure and expression pattern in ontogeny and adult in response to LPS stimulation also investigated.

1 Material and Methods

1.1 Database mining and cloning for *Xenopus* PGRP-L (XtPGRP-L)

To search for possible PGRP homologues, the reported PGRP protein sequences from *Drosophila* and mammals were used to perform tBLASTp search in the *Xenopus* genome database (<http://www.ensembl.org>). The obtained DNA sequence was then analyzed for predicted transcripts using GenScan at the Massachusetts Institute of Technology (<http://genes.mit.edu/GENSCAN.html>). To determine the correct reading frame of each exon, the BLAST program was used to maximize the in-frame translated sequence. A PGRP-L was identified in the database.

To obtain the full length of the identified XtPGRP-

L gene, total RNA was extracted from *Xenopus* liver using Trizol reagent (Invitrogen, USA). Approximately 500 ng total RNA was used as the template for the synthesis of first strand cDNA by reverse transcriptase using superscript II reverse transcription system (Invitrogen, USA). The full-length cDNA sequence of XtPGRP-L was amplified by random amplification of cDNA ends (RACE). The 5' end region of the PGRP-L was amplified by nested PCR with two primer pairs of UPM/PGRP-LRout (1st round PCR) and UPM/PGRP-LRin (2nd round). The gene specific primers used for 3' RACE PCR were UPM/PGRP-LFout (1st round PCR) and UPM/PGRP-LFin (2nd round). The PCR was carried out as per the following: in 25 µL solution containing 125 µmol of each dNTP, 0.2 µmol of each primer, 2.5 µL 10 × Taq buffer, 12 U Ex Taq polymerase, 18.1 µL sterile H₂O and 1 µL cDNA template, according to the standard protocol, and under the following conditions: an initial cycle of denaturation step at 94 °C for 5 min, followed by 8 cycles of amplification at 94 °C for 30 s, 64 °C for 30 s, 72 °C for 1 min, and 30 cycles at 94 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 10 min. A total of 10 µL of product was size-fractionated by 1.5% (W/V) agarose gel electrophoresis and stained with ethidium bromide. The desired PCR products were isolated using the Omega agarose purification kit, and cloned into pMD18-T vectors (TaKaRa) according to manufacturer's instructions, before being transformed into *Escherichia coli* DH5α competent cells and plated on selective agar plates containing 100 µg/mL of ampicillin. Positive colonies were grown overnight, and then determined using specific primers under the same cycle of PCR, before being sequenced using dideoxy chain-termination on an automatic DNA sequencer (ABI Applied Biosystems Mode 377). All primers used are listed in Tab.1.

Tab. 1 Primer sequences

Name	Sequences (from 5' to 3')	Annealing temperature(°C)
UPM	CTAATACGACTCACTATAGGGC	52
PGRP-LRout	CTCCACCTGCTCAGAGTCTGTTC	66
PGRP-LRin	AGCAGTGAGCGAGTAATGTTGGTT	63
PGRP-LFout	TGTATGAGGGACGTGTTTGAACA	65
PGRP-LFin	GATTCCATTCTGGCTTTGGTGAAG	63
PGRP-LF	GATGGAAAGCAGGAACAGA	
PGRP-LR	GAGACAGAGTCCCAGCATC	54
Actin-F	GGTCGCCCAAGACATCAG	
Actin-R	GCATACAGGGACAACACA	54

1.2 Sequence analysis of XtPGRP-L

The XtPGRP-L amino acid sequence was deduced using the Expasy translation tool (<http://ca.expasy.org/tools>). The alignment between the amino acid sequence of XtPGRP-L and other known PGRPs was analyzed using the CLUSTAL W program (version 1.83) (Thompson et al, 1994) and decorated using GeneDoc (<http://www.nrbsc.org/gfx/genedoc/index.html>). Identities between PGRP sequences were determined using the Megalign program within DNASTAR. A phylogenetic tree was constructed using the neighbor-joining (N-J) method within the Mega4.0 software program (<http://www.megasoftware.net/mega.html>) and all sequences were obtained from the NCBI database and are listed in Tab. 2. The 2-D structure of XtPGRP-L was predicted at PSIPREDView (<http://bioinf.cs.ucl.ac.uk/psipred/psiform.html>) (Jones, 1999; McGuffin et al, 2000). The 3-D structure of XtPGRP-L was constructed in the SWISS-MODEL protein modeling server (<http://expasy.org/swissmod/SWISS-MODEL.html>). A suitable structural template for XtPGRP-L, *Drosophila melanogaster* (Dm) PGRP-Lb (PDB file code: 1OHT-A), was identified by a BLAST search as implemented in the SWISS-MODEL protein modeling server (<http://expasy.org/swissmod/SWISS-MODEL.html>) (Arnold et al, 2006). The automatic sequence alignment obtained was used for homology modeling in SWISS-MODEL (Schwede et al, 2003), and the resultant theoretical model was displayed and analyzed with Swiss-PDB Viewer. The model picture was drawn using the Rasmol program (<http://www.umass.edu/microbio/rasmol/Rasmol>).

Tab. 2 Peptidoglycan recognition protein sequences used for phylogenetic tree construction and multiple sequence alignment

Species	Protein	GenBank accession No.	Species	Protein	GenBank accession No.
<i>Homo sapiens</i> (H.m)	PGRP1	O75594	<i>Xenopus tropicalis</i> (X.e)	PGRP-S	NP_001025626
	PGRP-L	AAL05629		PGRP-L	EF095491
	PGRP-I α	NP_443123		PGRP-SC2	XP_699934
	PGRP-I β	Q96LB8	<i>Danio rerio</i> (D.r)	PGLYRP6	ABE01406
<i>Macaca mulatto</i> (Ma.m)	PGRP-L	XP_001111895		PGRP2-A	ABE01404
	PGRP1	NP_033428		PGRP-SB1	CAD89129
<i>Mus musculus</i> (M.m)	PGRP-L	NP_067294	<i>Drosophila melanogaster</i> (D.m)	PGRP-SC1B	CAD89167
	PGRP-I β	NP_997130		PGRP-SC2	CAD89177
<i>Sus scrofa</i> (S.s)	PGRP-L	NP_998903		PGRP-LCx	AAM18530
<i>Canis familiaris</i> (C.f)	PGRP-L	XP_852999	<i>Drosophila simulans</i> (D.s)	PGRP-LCy	AAQ16306
<i>Rattus norvegicus</i> (R.n)	PGRP1	AAF73252		PGRP-LD	AAG36063
<i>Camelus dromedaries</i> (C.d)	PGRP	CAC19553		PGRP-LB	NP_731576
<i>Bos taurus</i> (B.t)	PGRP2	XP_588006		PGRP-LE	NP_573073
<i>Trichoplusia ni</i> (T.n)	PGRP	AAC31820		PGRP-SA	AAQ64766
<i>Enterobacteria phage</i>	T7 lysozyme	NP_041973			

1.3 Expression of XtPGRP-L mRNA in developing embryos and in different organs

To characterize the expression of XtPGRP-L in relation to *Xenopus* development and in different organs, developing embryos were collected at 3, 6, 15, 48, 72, 144, 216, and 360 h post-fertilization (hpf) for total RNA isolation (Dale & Slack, 1987). The RT-PCR was performed according to Liang et al. (2006), and the gene specific primers for RT-PCR were designed to the XtPGRP-L fragment and loading control primers were designed according to *Xenopus* β -actin. The primers used are listed in Tab.1.

To reveal the expression in different organs of adult *Xenopus*, heart, liver, lung, kidney, intestine, stomach and spleen were collected for total RNA preparation and

quantitative -PCR (Qi & Nie, 2008). All primers used are listed in Tab.1.

1.4 Preparation of polyclonal antibody against XtPGRP-L and Western blotting analysis

The 1 037 bp expression fragment of XtPGRP-L was obtained by one pair of primers containing *Kpn* I and *Hind* III digestion sites (Ex-PGRP-LF/R; Tab. 1). The PCR amplifications were performed at one cycle of 94 °C for 5 min, 33 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 60 s, with a final extension step of 72 °C for 10 min. Purified fragments were digested with *Kpn* I and *Hind* III, then ligated into the pQE-40 expression vector for recombinant protein construction, and transformed into *Escherichia coli* M15 competent cells (TAKARA) for protein expression. Fusion protein was expressed by

isopropyl- β -D-thiogalactopyranoside (IPTG) induction, and analyzed by SDS polyacrylamide gel (SDS-PAGE). Compared with the un-induced sample, a predominant extra protein band with molecular weight of about 58000 was observed in the IPTG induced samples. The recombinant protein was purified by affinity chromatography in a column of Ni^{2+} -charged resin (Novagen) according to manufacturer's instructions. To prepare the antibody, the purified recombinant protein was used to immunize rabbit. Briefly, the rabbit was first injected with 800 mg purified protein. Every two weeks, the rabbit was then immunized twice with half the initial amount of proteins. Two weeks after the last injection, the rabbit was bled and serum was collected and stored at -80°C .

To study the expression of XtPGRP-L at the protein level, heart, liver, spleen, lung, kidney, intestine and stomach were dissected and homogenized in buffer containing 1% SDS, 5% β -mercaptoethanol, 10% glycerol, 65 mmol Tris-HCl, pH 6.8 and 1 mmol PMSF. The suspension was vortexed and centrifuged at 10 000 g for 10 min, and the supernatant was retained. Protein concentrations were determined using Bradford protein assay (Biorad). Each sample, equivalent to 10 mg total protein, was run on 12% SDS-PAGE and subsequently transferred to PVDF membrane (Millipore) by standard procedures. The blotting membrane was blocked overnight at 4°C with TBS containing skimmed milk and 0.05% Tween 20, before being incubated for 2 h with rabbit antiserum (1:300). Subsequently, the membrane was further incubated for 1.5 h with Alkaline Phosphatase goat Anti-Rabbit IgG (Boster, China). After washing, the specific binding to recombinant protein was detected by NBT/BCIP (SABC).

1.5 Real-time quantitative PCR analysis of XtPGRP-L expression

Quantitative real-time PCR was performed to determine the expression of XtPGRP-L in normal frogs and those injected with LPS. Two groups of *Xenopus*, each containing three frogs, were injected intraperitoneally with phosphate-buffered saline (PBS, pH7.2) for the control and LPS (150 $\mu\text{g}/100\text{g}$ body weight) for the experiment, respectively. Twenty-four hours later, *Xenopus* were euthanized and tissues were collected for total RNA extraction and cDNA synthesis. The housekeeping gene β -actin amplified by actin-F/actin-R was used as the control. The primers used for real-time quantitative PCR analysis are listed in Tab. 1.

Quantitative real-time PCR was conducted on Chromo 4TM Continuous Fluorescence Detection from MJ Research. Amplifications were carried out at 20 μL volume, containing 1 μL of cDNA template, 10 μL of SYBR green Mix (Toyobo, Japan), 0.3 μL of each primer and 8.4 μL of ddH₂O. The PCR amplification was performed in triplicate wells, using the following conditions: 3 min at 95°C , followed by 40 cycles consisting of 30 s at 94°C , 25 s at 58°C and 25 s at 72°C . Melting curve analysis of the amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected.

Expression values were calculated using the relative standard curve method provided by the ABI Sequence Detection System software. Data analysis was done according to Purcell et al (2004). Statistical analyses (t -tests) were performed using Microcal origin 6.0 with $P \leq 0.05$ as the significance level.

2 Results

2.1 Sequence characteristics and phylogenetic analysis

A genomic scaffold_149 of 2.34 Mb in size was identified from *Xenopus* genome database. About 129 transcripts were predicted using the FGENESH program. Using Netblast analysis, the transcript that shared high identity with PGRP-L was verified. The XtPGRP-L cDNA sequence contained a 40 bp 5'-untranslated region (UTR), a 1 491 bp open reading frame, and a 150 bp 3'-UTR with a discernable poly (A) addition signal (AATAAA) (GenBank accession no. EF095491). The genome structure is conserved in *Xenopus*, zebrafish, mouse and human PGRP-L, which all contain five exons and four introns of similar size. Typical intron splice motifs were observed at the 5'(gt) and 3'(ag) ends of each intron. Putative XtPGRP-L possessed 497 amino acids, with a calculated molecular mass of 55 000 and an isoelectric point of 8.11. Analysis by SignalP showed that XtPGRP-L contained an 18-aa signal peptide (MAKVLLILLCTLCAMAVA). Similar to human, mouse and zebrafish PGRP-L, the XtPGRP-L also possessed a transmembrane domain (Fig.1).

Comparing the PGRP-L genes with PGRP-L from other vertebrates showed that all PGRP-L genes contained a conserved PGRP domain of approximately 160 amino acids (Fig.2). The identity between XtPGRP-L and other reported PGRP-L ranged from 31.3 % – 66.7%. Further analysis showed that this domain can be subdivided into three PGRP domains (I, II and III). The

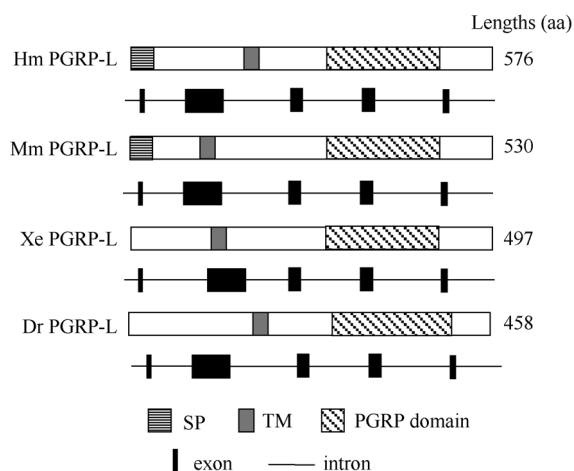


Fig. 1 Comparing the XtPGRP-L protein and gene structure with other vertebrates' long type PGRPs

Putative PGRP domains, signal peptides (SP) and transmembrane domains (TM) are indicated. Hm: *Homo sapiens*; Mm: *Mus musculus*; Xe: *Xenopus tropicalis*; Dr: *Danio rerio*.

three domains in XtPGRP-L had 8.3% to 51.2% identities, while domains II and III had the highest

(51.2%). The three PGRP domains in XtPGRP-L shared 52.6%, 87.5% and 78.0% identities with human counterparts, respectively. Although the identity between XtPGRP-L and T7 lysozyme (27.4% identity) was low, the positions of the amino acids needed for enzyme activity in T7 lysozyme, such as His-17, Gly40, Trp41, Tyr46, Arg60, His122 and Cys-130, were conserved (Fig. 2). The 2-D structure of XtPGRP-L predicted by PSIPREDView showed that XtPGRP-L was an α -helix/ β -strand mixed protein containing four α -helices and seven β -strands (Fig. 3a). The 3-D structure of XtPGRP-L was predicted by comparative modeling using *Drosophila melanogaster* PGRP-Lb (PDB file code: 1OHT-A) as a template. The model obtained from SWISS-MODEL was in accordance with the 2-D structure and showed that XtPGRP-L contained four α -helices and seven β -sheets. Compared with the Dm PGRP-LB (GenBank accession no. NP_731576), XtPGRP-L possessed a conserved structure: β 2- β 3- α 1- β 4- β 5- β 6- α 2- β 7- α 3. But, the XtPGRP-L has another α -helix (α^*) and β -sheet (β^*) (Fig. 3b).

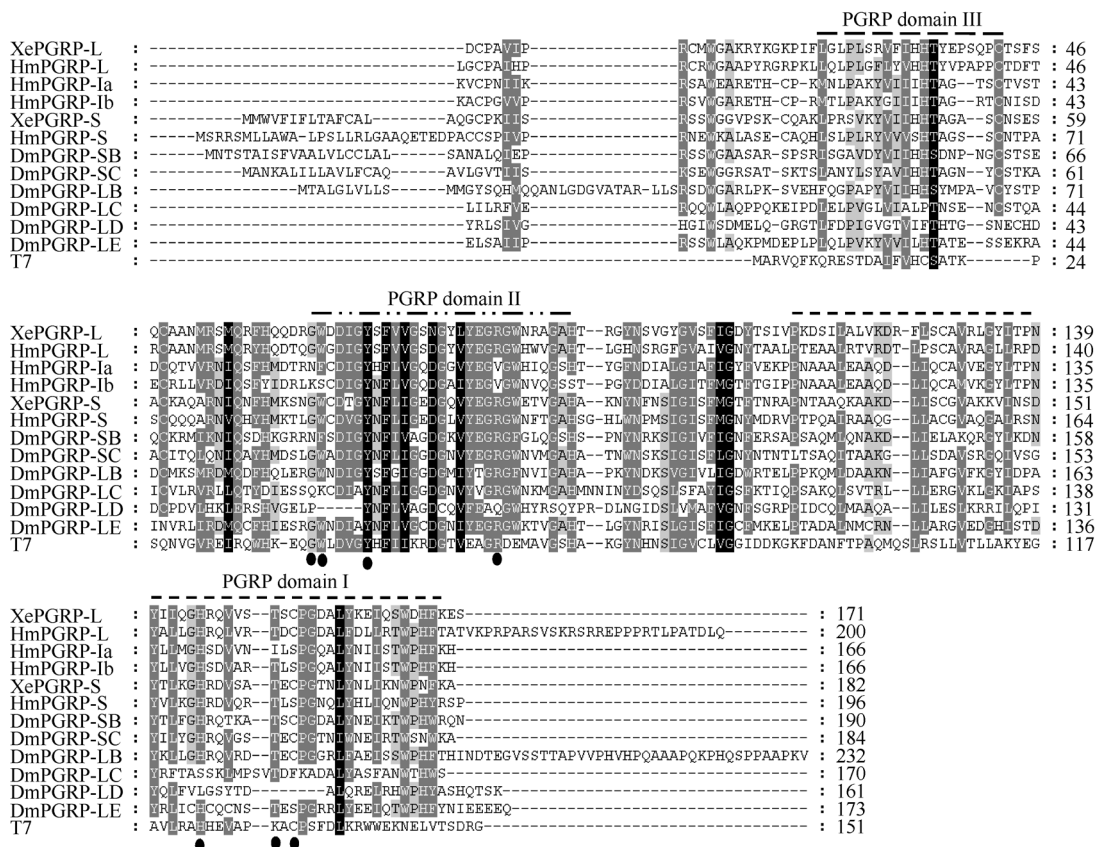


Fig. 2 Multiple alignment of XtPGRP-L with other PGRPs

Amino acid sequences were extracted from GenBank, and the alignment was produced with CLUSTL W. The three levels of shading indicate 100%, 80%, or 60% similar amino acids decorated with the GeneDoc program. The amino acids needed for T7 amidase are marked with dots. The GenBank accession no. of all sequences used are listed in Tab. 2.

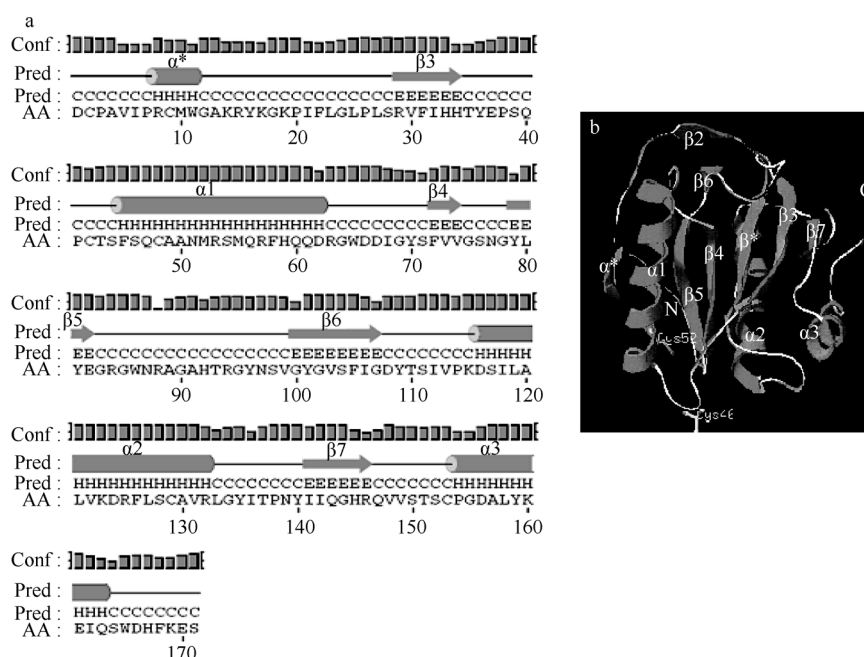


Fig. 3 The predicted secondary structure and homology modeling of XtPGRP-L

a) 2-D structure of XtPGRP-L. The conserved structures for all PGRPs are indicated as α_1 -3 and α_3 -7. The α^* is the specific region of XtPGRP-L. b) The 3-D structure of XtPGRP-L. The cysteines for the S-S bridge were labelled according to the Dm PGRP-Lb as cys46, cys52.

To further analyze the evolutionary relationship between XtPGRP-L and other PGRPs, an unrooted phylogenetic tree was constructed using the Neighbor-Joining methods. The XtPGRP-L was grouped closely with other vertebrate PGRP-L with high bootstrap values (about 99%). Surprisingly, the vertebrate PGRP-L was not clustered with the long type PGRP from *Drosophila melanogaster* (Fig.4). A previous study showed that PGRP-S may be the oldest PGRP type and evolved PGRP-L much later (Dziarski, 2004; Qi et al, 2010). Thus, the *Drosophila melanogaster* and vertebrates PGRP-L may evolve from a common PGRP-S ancestor gene and expanded by gene duplication.

2.2 Expression of XtPGRP-L gene in developing embryos and normal adults and in response to LPS stimulation

Using the XtPGRP-L specific primers, XtPGRP-L mRNA was not detected in early development stages (3 - 48 h) until 72 h (Fig. 5a). In normal adult *Xenopus*, XtPGRP-L mRNA was detected in liver, lung, intestine, stomach, heart, spleen and kidney (Fig. 5b, d). The XtPGRP-L was detected at the protein level in all organs examined by Western blotting. As shown in Fig. 5c, an immunoreactive band at about 55 000 was observed.

The XtPGRP-L expression pattern after LPS stimulation was examined using quantitative real-time PCR. The XtPGRP-L expression was obviously up-

regulated in the heart, liver, spleen and intestine after LPS treatment, with of 3-, 26- 7- and 33- fold increases, respectively. However, significant changes were not detected in the lung, kidney, and stomach (Fig. 5e).

3 Discussion

The present work described for the first time a long type PGRP from *X. tropicalis* by analyzing its genome database. The genomic structure of this PGRP and its expression pattern were analyzed.

The XtPGRP-L was 497-aa in length and contained a conserved genome structure, as in human, mouse and zebrafish. Furthermore, the XtPGRP-L also contained a highly conserved PGRP domain and shared high sequence identities with mammalian long type PGRP (eg. 66.7% with *Mus musculus*; 65.3% with *Homo sapiens*). The phylogenetic tree showed that XtPGRP-L grouped closely with PGRP-L from other vertebrates. These results confirmed that the cloned gene belonged to the long type PGRP. The predicted 3-D structure for the XtPGRP-L showed that the mixed β -sheet of six strands and three α -helices that formed the protein core was conserved in evolution. Furthermore, the Cys, Tyr and Thr residues located in β_3 - α_1 , α_1 - β_4 and β_7 - α_3 loops, respectively, are essential for PGN recognition (Reiser et al, 2004).

In T7 lysozyme, His-17, His-122, and Cys-130 are

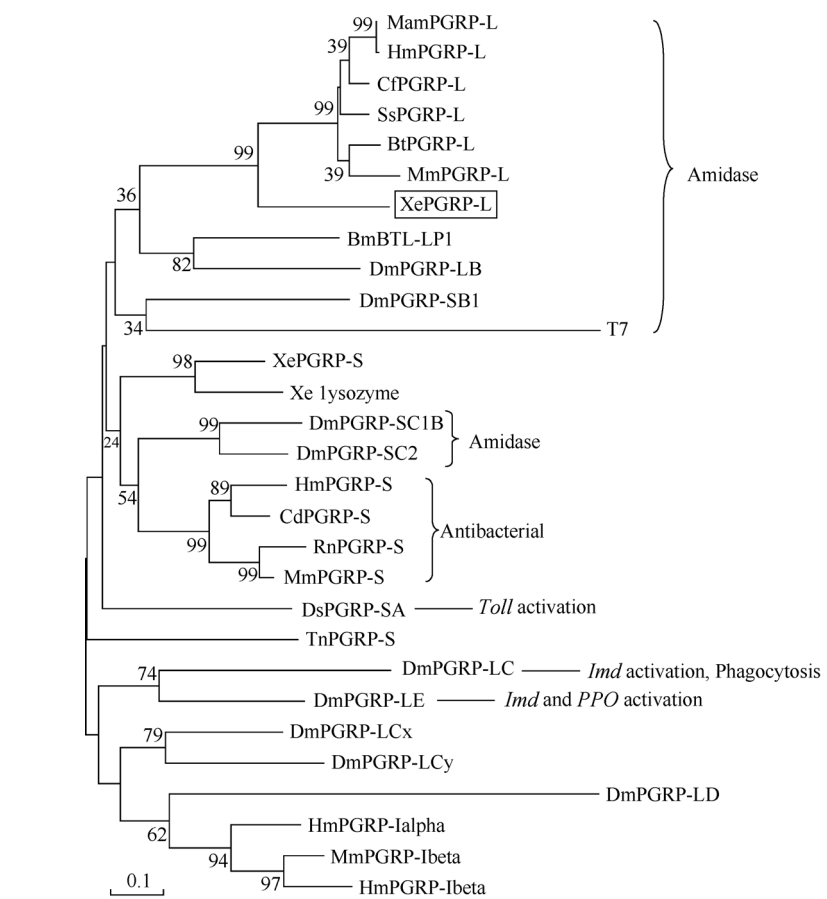


Fig. 4 An unrooted phylogenetic tree constructed by the neighbor joining from amino acid sequences of PGRPs together with the XtPGRP-L gene
The number indicates the bootstrap confidence values obtained for each node after 1000 replications. The GenBank accession no. of all sequences used are listed in Tab. 2.

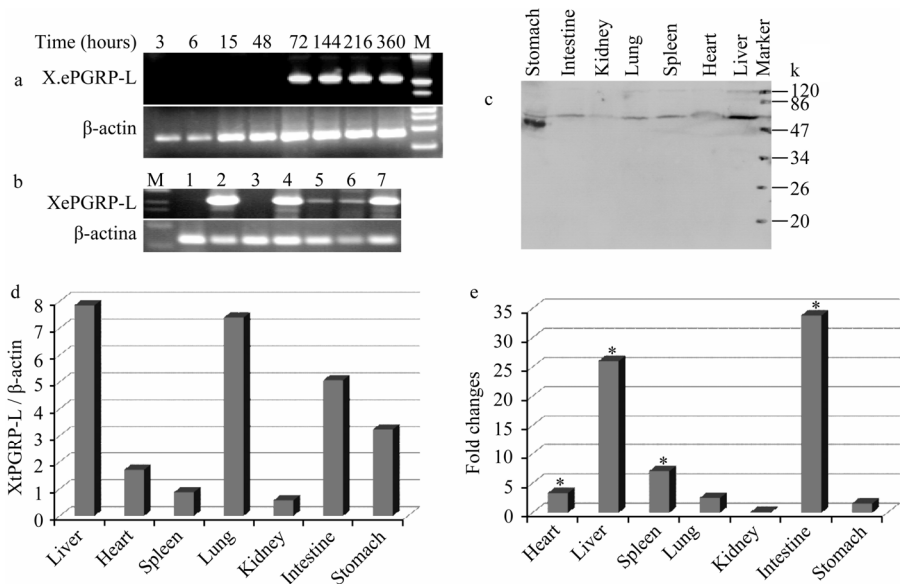


Fig. 5 Expression pattern of the XtPGRP-L gene in developing embryos, tissues of normal *Xenopus* and LPS stimulated *Xenopus*
(a) Expression of XtPGRP-L in developing embryos. (b) Tissue distribution of XtPGRP-L in normal *Xenopus*. β-actin was used as a control for the amount and quality of cDNA. Lines 1 to 7 represent spleen, liver, kidney, intestine, gall bladder, fat body, and stomach, respectively. (c) Western blot analysis of the XtPGRP-L expression in normal *Xenopus* tissues. (d) Quantitative PCR analysis of the XtPGRP-L expression in normal *Xenopus* tissues. (e) Quantitative PCR analysis of the XtPGRP-L expression change after LPS stimulation. Asterisks indicate that the copy number is significantly higher than that of the corresponding control sample (**P* < 0.05).

Zn²⁺ binding ligands, and Tyr-46 and Lys-128 are needed for enzymatic activity. Site directed mutagenesis of T7 lysozyme has shown that activity is retained when Lys-128 is replaced by Thr (Cheng et al, 1994). Divalent cations are required for the amidase activity of human PGRP-L, and Zn²⁺ fully restores its amidase activity. Among of four mammalian PGRPs, only PGRP-L has all amino acids needed for the amidase activity of T7 lysozyme conserved. In human PGRP-L, the Zn²⁺ binding amino acids and Cys419 are required for the amidase activity, whereas the three other amino acids, needed for the activity of T7 amidase, are not required (Wang et al, 2003). The alignment of the XtPGRP-L with T7 lysozyme and other PGRPs showed that residues needed for T7 lysozyme activity, were found in XtPGRP-L and other PGRPs proved to be amidase (Fig.3) (Steiner, 2004). This analysis suggests that XtPGRP-L might also be an amidase.

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