cDNA CLONING AND EXPRESSION CHARACTERIZATION OF LYSOZYME GENE IN TWO FRESHWATER PRAWN

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Abstract: Total RNAs were isolated from haemocytes of *M. rosenbergii* and *M. nipponense*. The cDNAs encoding lysozyme were amplified by RT-PCR. The amplified cDNA fragments were inserted into pGEM-T vector. Sequence analysis revealed that both of the two freshwater prawn lysozyme cDNAs contain an open reading frame (ORF) of 477nt, which encodes 158 amino acid residues, including 140 residues of mature peptide and 18 residues of signal peptide. The nucleotide and amino acid sequence identity between the two prawn cDNAs is 99.4% and 98.1%, respectively. The two prawn cDNAs possess high identity with marine shrimp cDNAs, too. Their nucleotide and amino acid sequence identity is above 83.0% and 80.0%, respectively. The prawn lysozymes are presumed to be the non-calcium binding family of chicken-type lysozyme because they have two conserved catalytic sites Glu^{51} and Asp^{68}, as well as eight structural Cys residues, which are highly conserved among the species of chicken-type lysozymes but lack of three Asp residues at the site 101, 106 and 107, which are conserved sites in calcium-binding chicken-type lysozyme. The expression pattern of lysozyme gene of *M. rosenbergii* infected with *Vibrio* spp was analyzed by Northern Dot Blot with biotin-labeled probe produced by PCR. The result showed that the expression levels of lysozyme were up-regulated in eye, muscle, gill, hepatopancreas, intestine. Among them the lysozyme mRNA level in hepatopancreas was the highest which was 560% of that of the control group. And the lysozyme mRNA levels in hepatopancreas varied at different infection time: The lowest level was at 3h post-infection, the highest was at 24h post-infection, which was about 430% of that of the control group. At 48h post-infection, the mRNA level decreased slightly, but still higher than that of the control group (about 330%). The up-regulation of lysozyme gene expression observed in *Vibrio* infected *M. rosenbergii* suggested that freshwater prawn lysozyme gene is undoubtedly related to the non-specific immune defense and hepatopancreas may also play an important role in prawn immune mechanism.

Key words: *Macrobrachium rosenbergii; M. nipponense;* Lysozyme; Gene cloning; Expression pattern
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Lysozyme (EC3.2.1.1.17) is one of the most important non-specific immune factors in the organism. The biological function of the lysozyme is believed to be self-defence from bacterial infection by hydrolyzing β-1, 4 linked glycosidic bond of the peptidoglycan on the bacterial cell wall[1]. Lysozyme are classified into six types: chicken-type lysozyme (c-type), which includes calcium-binding lysozyme and non-calcium binding lysozyme; goose type lysozyme (g-type), plant lysozyme, bacterial lysozyme, T_4 phage lysozyme (phage type), and invertebrate lysozyme (i-type)[2].

G-type lysozyme is most abundant, being distributed in many of vertebrates from fishes to mammals and also found in insects such as moths and fies[2]. There was a debate about the lysozyme activity of the shrimp haemocytes previously. The haemocytes of the *Litopenaeus vannamei* was found to possess lysozyme activity[3], while the haemocytes of the *Penaeus setiferus* was not[4]. However more and more researches suggested that the lysozyme is one of the most important immune factors in shrimps[5]. Liu *et al* reported the lysozyme activity of the *L. vannamei* and *Penaeus chinese*[6—7]. Nappi *et al* have isolated the lysozyme with antibacterial activity from the haemocytes of *L. vannamei*[8]. Rojtinnakon has found that the lysozyme was one of the most important antibacterial proteins among the proteins which were deduced

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from the immune related genes of WSSV infected *Marsupenaeus japonicus*[9]. Recently Rogerio *et al* have cloned the cDNA of lysozyme of *Penaeus vannamei*, and detected the lysozyme activity in haemocytes[10]. Hikima *et al* have cloned the lysozyme gene of *M. japoncius*, and expressed the lysozymegene in insect cells, the recombinant lysozyme displayed lytic activities against several *Vibrio* species and fish pathogens[11]. Lysozyme cDNAs of *Penaeus semisulcatus*, *P. monodon* and *L. vannamei* have been isolated recently in our labortary. The lysozyme cDNA of *P. monodon* have been expressed in *E. coli*, and the recombinant lysozyme had *in vitro* potent lytic activities to *Micrococcus lysodeikticus* and *Vibrio alginolyticus*, which confirmed the immune defense funetion of the shrimp lysozyme[12—13]. In this paper the lysozyme genes of the *M. rosenbergii* and *M. nipponense*, the two freshwater prawns, were cloned, and the tissue expression pattern of lysozyme gene in the infected *M. rosenbergii* was studied.

1 Materials and Methods

1.1 cDNA cloning of the *M. rosenbergii* and *M. nipponense* lysozyme genes Anticoagulant was prepared referring to the method of Gross[14]. The haemocytes were taken from the dorsal sinus located at the base of the first abdominal segment by syringe with anticoagulant and immediately centrifuged 30s at 12, 000g to separate the blood cells from plasma. The total RNAs were extracted according to the protocol of the SV Total RNA Isolation Kit (Promega Company). The quality of the total RNA was detected by 1.5% agarose gel electrophoresis.

Primers were designed according to the nucleotide sequences of lysozyme registered in GenBank (AF425673, AF321519). The upstream primer is P_1: 5' GTGATAATACTTGAGTCATAGAAATG3', downstream primer is P_2: 5' CTAGAACGGGAAGACAGAGTTGG3'. Using the oligo dT-adaptor primer provided by TaKaRa RNA PCR Kit (AMV), first strand cDNA was synthesized by reverse transcription. The reaction conditions were: 30℃, 10min; 50℃, 30min; 95℃, 2min; 5℃, 5min. The cDNA was then amplified by PCR using P_1 and P_2 as primers. PCR products were checked by agarose gel electrophoresis.

The PCR products were purified by Agarose Gel DNA Purification Kit (TaKaRa), and cloned into pGEM-T Vector (Promega Company). Recombinant clones were identified and proliferated. Plasmids DNA were extracted by alkline lysis method[15] and sequenced by ABIPRSM^{TM}377. Sequences were analyzed by the vector NTI suit 6.0 and homology was searched with the BLAST program at NCBI.

1.2 Immune challenge and extraction of total RNAs of different tissues The freshwater prawns, *M. rosenbergii* about 18—25 g in body weight were obtained from prawn farm, and cultivated in aquarium, equipped with air-pump. The *Vibrio*. spp was cultivated for 16h, and then diluted to 6×10^7 cells/mL. 50μL of the *Vibrio* were injected into the prawns' abdominal muscle. Total RNAs of haemocytes, eyes, muscle gill, hepatopancreas, and intestine were extracted 6h after infection according to the protocol of SV Total RNA Isolation Kit. Total RNAs of hepatopancreas of the infected and control (injected with saline) prawns were also collected at 3h, 6h, 12h, 24h and 48h. The quality of total RNAs was detected by agarose gel electrophoresis.

1.3 Northern Dot Blot analysis of the tissue expression of lysozyme mRNA Using the recombinant plasmid of *M. rosenbergii* lysozyme gene as a template, P_1 and P_2 as primers, the biotin-labeled probe of lysozyme gene was synthesized by PCR. The ratio of the dTTP to the biotin-dTTP was 3∶1 in the reaction system. Reaction conditions were: 1 cycle of 94℃, 3min; 30 cycles of 94℃, 30s; 56℃, 45s; 72℃, 1min; 1 cycle of 72℃, 8min.

Two primers were designed according to the conserved sequences of 18S rRNA of other decapoda species registered in GenBank (AF235960, AF235959, AF235961): 18S-P_1: 5' AGGGAAGAGCGCTTTTATTA3', 18S-P_2: 5' CTATTGGAGCTGGAATTACC3'. Genomic DNA from hepatopancreas was extracted using alkaline lysis method. The expected PCR products were purified and cloned into the pGEM-T vector and sequenced. The 18S rRNA recombinant plasmid was used as a template to synthesized biotin-labeled probe of 18S rRNA by PCR as described above. The two gene probes were purified, qualified by spectrophotometer, and denatured by incubating in 95℃ for 10min, and immediately placed on ice before use.

Total RNAs from 10 individuals were mixed. 5μg total RNAs of different tissues from control group and infection group were denatured and dotted onto the membranes (Hybond-N^+). The membrane with RNA was baked for 3min in the microwave oven (750W) and were prehybridized with salmon sperm DNA at a final concentration of 100μg/mL at 42℃ for 2h. Then the membrane was hybridized at 42℃ for 18h with biotin-labeled lysozyme gene probe in hybridization

buffer containing 50% formamide. Total RNAs of hepatopancreas of 3h, 6h, 12h, 24h, 48h post-infection were also treated as above.

The membrane was washed and detected according to the protocol of the Detector™ AP Chemiluminescent Blotting Kit (KPL Company). In brief, after hybridization the membrane was washed 2 × 15min in 2 × SSPE (with 0.5% SDS) at room temperature, then washed 2 × 30min at 55 ℃ in 0.2 × SSPE (with 0.5% SDS), and finally washed in 1 × SSPE 5min at room temperature. The membrane was blocked with 1 × Block Solution for 45min, then incubated for 30min in fresh blocking solution with AP-SA (Alkaline phosphatase-labeled streptavidin, 1∶10000). The membrane was washed in 1 × Wash Solution 3 times for 5min each, and rinsed 2 × 2 min in 1 × Assay Buffer. Then incubated stationary for 5 min in CDP-Star Chemiluminescent Substrate, and placed in a hybridization bag and exposed to X-ray film.

The membrane was stripped with 0.1% SDS solution and then hybridized with the biotin-labeled 18S rRNA gene probe using the same procedure.

1.4 Scanning and Statistics Signals were scanned and quantified by the FR-1000 (Analysis System of Biology Image). The signals' consistencies and areas were obtained, and their products represented the signal intensity. The signal intensity ratio of the lysozyme to 18S rRNA counted as the relative expression level of lysozyme referring to the methods of Destoumiex *et al* and Munoz *et al*[16—17].

2 Results

2.1 Cloning and characterization of lysozyme ORFs of two prawns

The specific fragment about 500nt in length in the amplified products was cloned and identified by endonuclease digestion (Fig. 1). The cDNAs from both *M. rosenbergii* and *M. nipponense* contain an open reading frames (ORF) of 477nt, which encodes 158 amino acid residues, including 140 residues of mature peptide and 18 residues of signal peptide. The deduced molecular weight of the mature peptide of lysozyme of *M. rosenbergii* was 16,278Da and pI was 8.79, while for *M. nipponense* those values were 16,245Da and 8.38, respectively. The accession numbers for *M. rosenbergii* and *M. nipponense* lysozyme gene in the GenBank are AY257549 and AY257550.

The nucleotide and deduced amino acid sequences identity between the two freshwater prawn lysozyme cDNA is 99.4% and 98.1%, respectively. The two prawn lysozyme cDNAs also possessed high identity with marine shrimp cDNAs, including cDNAs from *P. monodon*, *L. vannamei*, *P. semisulcatus*, *M. japonicus*. Their nucleotide and amino acid sequence identity was above 83% and 80%, respectively. While identity of the two prawn cDNAs with other species' such as fishes, mammals was 38% ~ 45% (Tab. 1).

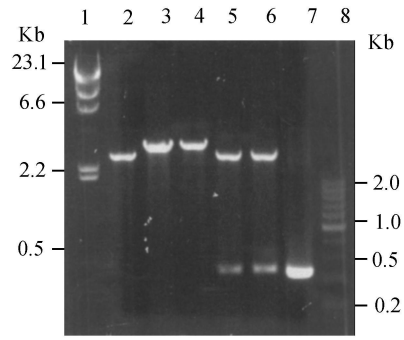

Fig. 1 The PCR products and identification of the recombinants
1. λ-DNA/*Hind* Ⅲ Marker; 2. T-Vector/*Pst* Ⅰ; 3. *M. rosenbergii* Recombinant/*Pst* Ⅰ; 4. *M. nipponense* Recombinant/*Pst* Ⅰ; 5. *M. rosenbergii* Recombinant/*Not* Ⅰ; 6. *M. nipponense* Recombinant/*Not* Ⅰ; 7. PCR product of *M. rosenbergii*; 8. 200bp DNA Marker

Tab. 1 Identities of the deduced amino acids sequence of the two freshwater prawn lysozyme cDNAs with e-type lysozyme of other species

	1	2	3	4	5	6	7	8
M. rosenbergii	98.7	91.1	88.6	81.6	45.0	38.6	41.4	39.9
M. nipponense	98.1	90.5	88.0	81.6	45.6	38.6	41.1	40.5

Note: 1. *Penaeus monadon* 2. *Penaues semisulcatus* 3. *Litopenaeus vannamei* 4. *Marsupenaeus japonicus* 5. *Oncorhynchus mykiss* 6. *Anopheles darlingi* 7. *Gallus gallus* 8. *Homo sapiens*

The deduced amino acid sequences of the lysozyme from the two freshwater prawns have two conserved catalytic sites Glu^{51} and Asp^{68}, as well as eight structure Cys residues which are highly conserved among many species of e-type lysozyme. The sequences around the catalytic site are quite conserved, too. Moreover both the lysozymes lack of Asp residues at the positions of 101, 106 and 107, which are conserved sites in calcium-binding e-type lysozyme. Therefore the two freshwater prawn lysozymes are presumed to belong to non-calcium binding family of e-type lysozyme (Fig. 2).

2.2 Tissue expression of the lysozyme in *Vibrio* infected *M. rosenbergii*

2.2.1 The lysozyme mRNA level in different tissues

Northern Dot Blot signals analysis indicated that 6h after infection by *Vibrio*, the lysozyme mRNA level increased in all the detected tissues except in haemocytes which decreased

slightly, and the highest level was in hepatopancreas, which was about 560% of that of the control group (Fig. 3).

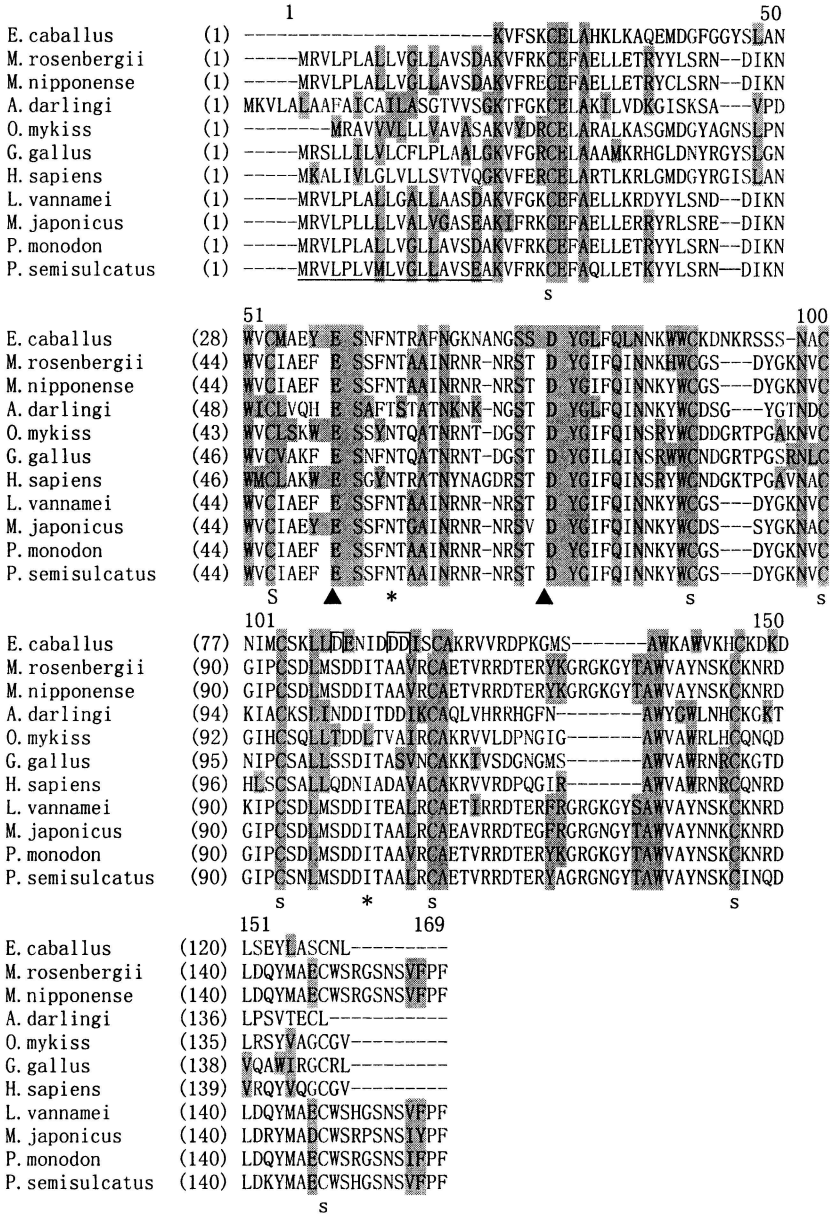

Fig. 2 Comparison of the deduced amino acids of the freshwater prawns and c-type lysozyme of other species

Note: The '▲' indicate the catalytic residues (Glu[51] and Asp[68]); 's' indicates the cysteins residues; '*' indicates the special activies sites; the underlined sequence indicates the signal peptides; the frames indicate the conserved calcium-binding sites. *Equus caballus* (2EQL); *Gallus gallus* (CAA23711); *Homo sapiens* (NP-000230); *L. vannamei* (AAN86086.); *M. rosenbergii* (AAP13357); *M. nipponense* (AAP13578); *Oncorhynchus mykiss* (AAG34564); *P. monodon* (AAN16375); *P. semisulcatus* (ANN860865); *M. japonicus* (BAC57467)

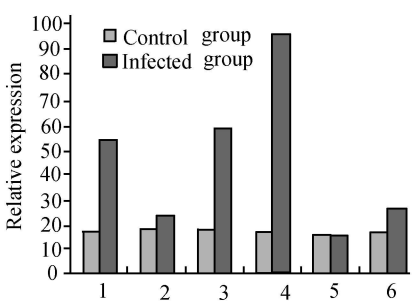

Fig. 3 Comparison of lysozyme gene expression between control group and 6h post-infected group

1. eye; 2. muscle; 3. gill; 4. hepatopancreas; 5. haemocytes; 6. intestine

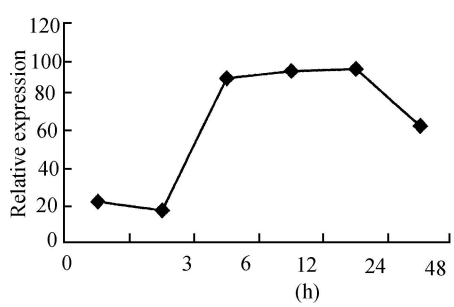

Fig. 4 Time-course analysis of lysozyme gene expression in hepatopancreas of *M. rosenbergii* infected with *Vibrio*

2.2.2 Time-course analysis of lysozyme expression in hepatopancreas The lysozyme mRNA level in hepatopancreas decreased slightly at 3h post-infection, but increased rapidly at 6h post-infection, which was about 400% of that of the control group. The lysozyme mRNA level in hepatopancreas at 24h post-infection was the highest (about 430%). At 48h post-infection, the mRNA level in hepatopacreas was decreased but still much higher than that of the control group (about 330%) (Fig. 4).

3 Discussion

The deduced amino acid sequence of two freshwater prawn lysozymes cloned, as well as other reported marine shrimps, containing 158 amino acid residues, are larger than the c-type lysozyme molecule of other species, for example, lysozymes from *Homo sapiens*, egg white *Gallus gallus* and insects (*Samia cynthia ricini*) have about 150 residues. The missed or inserted residues in prawn lysozymes do not belong to the enzyme activity sites or the structural conserved sites, as showed in Fig. 2, so they are supposed to have no effect on the activity or the structure[12]. The deduced amino acid sequence of lysozymes cDNA we cloned have 9 extra residues at the c-terminus (HGSNSVFPF) similar to that of *P. vannamei*, which Rogerio *et al* suggested to be considered as the characteristic of marine invertebrate lysozyme[10].

The lysozyme mRNA levels in most of the checked tissues increased at 6h post-infection especially in the hepatopancreas, gill and eyes. High mRNA levels maintained in hepatopancreas from 6h to 24h, and still much higher than that of the control group at 48h post-infection. The increasement of the lysozyme expression level is undoubtedly related to the immune defense. Gross *et al* found that 4% —5% of the EST gained from the hepatopancreas of *L. vannamei* and *L. setiferus* were immunity related, so it is reasonable to postulate that hepatopancreas, an organ of haemolymph filteration and digestion, could play an important role in immune defense[10]. Gill is a breath organ of prawn, haemocytes obtain dissolved oxygen in the water through gill's thin respiratory epithelium. The high level of lysozyme in gill is essential to a tissue with such specific function and specific structure to defense pathogen. The lysozyme mRNA expressed in the intestine of the non-challenged individuals, and increased after being infected. These phenomena may support the viewpoint of Ito *et al*, who suggested that the biological function of lysozyme is important in their self-defense against bacterial infection as well as in digestion[2].

In haemocytes, the level decreased slightly than control group at 6h post-infection. The reason could be contributed to the specific response of the haemocytes to *Vibrio*, and a longer period was needed for it to return to normal level and then to increase. The same phenomenon was observed in antibacterial peptides mRNA levels in the haemocytes of the *L. vannamei* after being infected with *Vibrio*[16].

In conclusion, lysozyme ORFs of two freshwater prawns *M. rosenbergii* and *M. nipponense* were isolated and presumed to belong to non-calcium binding family of c-type lysozyme. The up-regulation of lysozyme gene transcription was observed in most detected tissues of *Vibrio* infected *M. rosenbergii*. The time-course expression pattern of hepatopancreas suggested that hepatopancreas also plays an important role in freshwater prawn immune mechanism.

Acknowledgments

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两种沼虾溶菌酶基因 ORF 的克隆和罗氏沼虾溶菌酶基因的组织表达

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摘要: 分别提取罗氏沼虾和日本沼虾血细胞总 RNA, RT-PCR 扩增获得特异性 cDNA 片段, 纯化后克隆到 T 载体上。序列测定表明所克隆的两种沼虾溶菌酶基因的开放阅读框(ORF)为 477bp, 共编码 158 个氨基酸, 包括溶菌酶成熟肽 140 个氨基酸残基和信号肽 18 个氨基酸残基。同源性分析表明, 罗氏沼虾和日本沼虾溶菌酶基因的碱基序列及推测氨基酸序列高度同源, 分别为 99.4% 和 98.1%。两种沼虾溶菌酶基因的碱基序列和推测氨基酸序列与 GenBank 上其他对虾溶菌酶的同源性达 83.0% 和 80.0% 以上。两种沼虾溶菌酶都具有 e-型溶菌酶典型的两个酶活性位点(Glu^{51})和(Asp^{68}), 以及 8 个保守结构氨基酸残基 Cys, 且在 101、106 和 107 位上缺少 Asp, 因而推测本实验所克隆的两种沼虾溶菌酶基因属 e-型溶菌酶基因的非钙结合亚型。以 PCR 法制备罗氏沼虾溶菌酶基因的生物素标记探针, 斑点杂交检测感染弧菌后溶菌酶基因 mRNA 在各组织中的转录水平, 结果表明受感染 6h 后在眼、肌肉、鳃、肝胰腺、肠管中的表达量均有升高, 其中在肝胰腺中的表达量最高, 约为对照组的 560%。在不同感染时间里, 肝胰腺中该基因表达量有较大的变化: 感染后 3h 表达量最低, 24h 后表达量升至最高, 大约为对照组的 430%, 48h 时的表达量又有所下降, 但仍明显高于对照组(约为 330%)。受弧菌感染后罗氏沼虾溶菌酶基因转录的上调证明溶菌酶基因在非特异性免疫中的直接作用, 同时表明肝胰腺可能在沼虾的免疫防御过程起重要作用。

关键词: 罗氏沼虾; 日本沼虾; 溶菌酶; 基因克隆; 基因表达