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博 士 学 位 论 文

两种石首鱼天然免疫因子（MIF 和 pglyrp2）的基因克隆、表达特性及生物学活性研究

Study on Gene Cloning, Expression and Bioactivity of Innate Immune Factors (MIF and pglyrp2) in Two Species of Croakers

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缩略语中英文对照表

缩写	英文	中文
aa	Amino acid	氨基酸
Amp	Ampicillin	氨卡青霉素
BLAST	Basic local alignment search tool	基本局域联配搜寻工具
bp	Base pair	碱基对
Bis	Bisacrylamide	N'-甲叉丙烯酰胺
BSA	Bovine serum albumen	牛血清蛋白
cDNA	Complementary DNA	互补脱氧核糖核酸
DDT	D-DOPAchrome tautomerase	D-多巴色素互变异构酶
DEPC	Diethyl pyrocarbonate	焦碳酸二乙脂
DTT	Dithiothreitol	二硫苏糖醇
EB	Ethidium bromide	溴化乙啶
EDTA	Ethylene diamine teraacetic acid	乙二胺四乙酸
ELISA	Enzyme linked immunosorbent assay	酶联免疫吸附实验
EST	Expressed sequence tag	表达序列标签
GSH	Reduced Glutathione	还原型谷胱甘肽
IBs	Inclusion bodies	包涵体
IPTG	Isopropyl-β-D-Thiogalactopyranoside	异丙基硫代-β-D-半乳糖苷
kDa	Kilodalton	千道尔顿
KI	Killing index	杀伤指数
LB	Luria-Bertani medium	LB 培养基
LPS	Lipopolysachcaride	脂多糖
MBL	Mannose-binding Lectin	甘露糖结合凝集素
MIF	Macrophage migration inhibitory factor	巨噬细胞移动抑制因子
mRNA	Messenger ribonucleic acid	信使 RNA
NCBI	National center for biotechnology information	美国国家生物信息中心
NO	Nitric oxide	一氧化氮
ORF	Open reading frame	开放阅读框
PAGE	Polyacrylanide gel electrophoresis	聚丙烯酰胺凝胶电泳
PAMPs	Pathogen associated molecular patterns	病原菌关联的分子模式
PBS	Phosphate buffer saline	磷酸盐缓冲液
PCR	Polymerase chain reaction	聚合酶链式反应
PGN	Peptidoglycan	肽聚糖
pglyrp	Peptidoglycan recognition protein	肽聚糖识别蛋白
PGRP	Peptidoglycan recognition protein	肽聚糖识别蛋白
pNPP	ρ-nitrophenyl phosphate, disodium	对硝基苯磷酸二钠
PRR	Pattern recognized receptors	模式识别受体
RACE	Rapid amplfication of cDNA ends	快速扩增 cDNA 末端
RT	Reverse transcription	反转录

缩写	英文	中文
SDS	Sodium dodecyl sulfate	十二烷基硫酸钠
SDS-PAGE	SDS-polyacrylanide gel electrophoresis	SDS-聚丙烯酰胺凝胶电泳
Smart	Switching mechanism at 5’ end of RNA transcript	RNA 转录本 5’末端转换机制
TAE	Tris-acetic acid-EDTA buffer	Tris-乙酸 EDTA 缓冲液
TLR	Toll like receptor	Toll 类受体
TPOR	Thiol-protein Oxidoreductase	硫醇蛋白氧化还原酶
U	Unit	活性单位
UTR	Untranslated region	非翻译区

摘 要

鱼类所生活的水环境中充斥着大量的病原菌，引起的疾病每年都给海水鱼类养殖带来了巨大的损失，本论文以大黄鱼 (*Pseudosciaena crocea*) 抗病遗传育种为研究背景，为了解哈维氏弧菌 (*Vibrio harveyi*) 感染大黄鱼过程中涉及的病原菌识别和炎症反应等免疫应答相关的分子机制，开展了两种石首鱼天然免疫因子的基因克隆、表达特性及生物学活性研究。

巨噬细胞移动抑制因子 (Macrophage migration inhibitory factor, MIF) 作为多功能的炎症细胞因子，在炎症疾病的发生发展过程中起着关键的调节作用，但是，关于鱼类MIF的功能研究和炎症致病机理的研究却报道甚少；肽聚糖受体是目前分子免疫学的研究热点之一，其中，肽聚糖识别蛋白II (pglyrp2) 是一类具有酰胺酶活性、能识别细菌细胞壁特有成份——肽聚糖的模式识别受体，Li (2007) 首次报道了斑马鱼 (*Danio rerio*) PGRP具有广谱的抗菌活性，这是PGRP家族重要的功能新发现。为此，本论文首先开展了两种石首鱼——大黄鱼和眼斑拟石首鱼 (*Sciaenop ocellatus*)——的天然免疫因子 (MIF和pglyrp2) 的基因克隆和序列分析的研究工作，然后围绕着大黄鱼开展了天然免疫因子MIF和pglyrp2基因的mRNA表达、蛋白表达纯化及生物学活性的研究，获得了如下结果：

1、克隆了大黄鱼与眼斑拟石首鱼MIF的cDNA和基因全长

本文采用同源克隆方法和RACE技术，从大黄鱼和眼斑拟石首鱼的肝脏中克隆了MIF cDNA序列。另外还以肌肉基因组DNA为模板，克隆了大黄鱼和眼斑拟石首鱼MIF基因全长，Genebank的登录号分别为FJ415099和FJ447489。

大黄鱼MIF和眼斑拟石首鱼MIF的cDNA全长都含有一个345 bp的开放阅读框，3’的末端都含有加尾信号；MIF的ORF都编码一个长115氨基酸的蛋白质，没有信号肽。表明鱼类MIF的分泌类似于哺乳动物的MIF，不是通过传统的蛋白分泌途径、经内质网向胞外分泌MIF蛋白的。另外，大黄鱼和眼斑拟石首鱼的MIF基因都含有3个外显子和2个内含子；序列比对发现，所有的外显子和内含子、以及ORF所编码的氨基酸序列在两种鱼的MIF中都十分保守，同一性都超过88%。氨基酸序列分析还表明，这两种鱼MIF都含有典型的硫醇蛋白氧化还原酶

(TPOR) 催化活性所需的CALC结构，预示着鱼类MIF可能具有TPOR活性。

2、克隆了大黄鱼与眼斑拟石首鱼pglyrp2的cDNA和基因全长

采用同样的实验方法，本研究还克隆了大黄鱼和眼斑拟石首鱼pglyrp2的cDNA和基因全长。大黄鱼pglyrp2 cDNA的Genebank登录号为FJ385025，pglyrp2基因的登录号为FJ385024；眼斑拟石首鱼pglyrp2 cDNA的Genebank登录号为FJ415100，pglyrp2基因的登录号为FJ415101。

大黄鱼pglyrp2和眼斑拟石首鱼的pglyrp2的cDNA全长都含有1446 bp的开放阅读框，都编码一个长482 aa的蛋白质，带有一个21aa的信号肽，表明它们是分泌蛋白。另外，大黄鱼和眼斑拟石首鱼的pglyrp2的基因含有4个外显子和3个内含子，基因序列十分保守，其中ORF的碱基差异仅3.24%。系统进化分析表明，鱼类pglyrp2间的亲缘关系相近，并与哺乳动物的pglyrp2在同一个进化分支上。氨基酸序列分析表明，这两种鱼的pglyrp2含有酰胺酶活性所需的关键残基，预示着鱼类的pglyrp2也可能具有酰胺酶的活性。

3、揭示了大黄鱼MIF和pglyrp2基因转录体在不同组织的分布

应用RT-PCR技术和Realtime PCR技术，检测了大黄鱼肝脏、性腺，鳃、脾脏、肌肉、肠、胃、头肾、心脏和脑等11个组织的MIF和pglyrp2的mRNA表达，发现大黄鱼MIF基因在11个组织中都有表达，其中在脑和肝中表达量较高，MIF转录体在多种组织的广泛存在表明鱼类MIF可能具有重要的生物学功能。大黄鱼pglyrp2基因在肝脏强烈表达，在性腺、肠、胃中弱表达，在其它组织中不表达，具有明显的组织特异性。另外，大黄鱼未受精卵中pglyrp2基因强烈表达，在胚胎和早期仔鱼阶段弱表达，pglyrp2 mRNA在发育早期的时序表达模式表明它可能在保护卵的免疫防御中具有重要作用。

4、揭示了大黄鱼MIF mRNA在哈维氏弧菌 (*Vibrio harveyi*) 攻毒后表达量的组织变化

哈维氏弧菌人工攻毒后，观察到大黄鱼出现了严重的皮肤溃疡，组织切片表明攻毒后大黄鱼的肝、头肾和肠等组织中发生了炎症病变。同时，荧光定量PCR分析表明，大黄鱼MIF mRNA在攻毒后大黄鱼肝脏中表达量极显著上调，在性腺中的表达量显著上调，结果似乎表明鱼类MIF与炎症相关疾病的致病过程相关。

5、研究了大黄鱼pglyrp2 mRNA在细菌人工感染下的组织表达变化

利用革兰氏阳性菌 (溶壁微球菌，*Micrococcus lysoleikticus*) 或革兰氏阴性菌(哈维氏弧菌) 人工感染大黄鱼，同时测定SOD酶的活性和利用Real time PCR检测mRNA表达量的变化。虽然哈维氏弧菌感染导致被感染大黄鱼出现了明显弧菌病症，而非病原菌溶壁微球菌并未引起大黄鱼的组织病变，但二者都引起了SOD酶活性的增加和肝脏等组织的mRNA表达量的增加。结果表明鱼类pglyrp2作为模式识别受体对细菌种类的识别没有特异性，并可能在介导激活免疫防御反应方面起着重要作用。

6、完成了 MIF 和 pglyrp2 的原核表达与蛋白纯化

根据已获得的大黄鱼 MIF 和 pglyrp2 的 cDNA 序列，成功构建了 pET-MIF、pET-pglyrp2 和 pET-pglyrp2 成熟肽的融合表达载体，在大肠杆菌中经 IPTG 诱导表达后，所获得的 MIF 融合蛋白主要以可溶的形式存在于超声波破碎菌体后得到的上清里，而所获得的 pglyrp2 融合蛋白与 pglyrp2 成熟肽融合蛋白主要以不可溶的形式存在于包涵体中。MIF 融合蛋白在非变性条件下经过亲和层析和浓缩脱盐纯化后，得到了纯化的单一条带蛋白，目的蛋白纯度高达 90%以上，MIF 融合蛋白的体外大量可溶性表达为后续开展 MIF 的生物学功能奠定了基础。而 pglyrp2 融合蛋白和 pglyrp2 成熟肽融合蛋白，在变性条件下，经过纯化后，大量析出沉淀，只有不到 6%的重组蛋白在透析后保持可溶状态，所获蛋白为开展多克隆抗体制备等免疫学研究奠定了基础。

7、首次阐述了鱼类MIF融合蛋白具有TPOR催化活性和刺激巨噬细胞产生NO的活性

实验表明大黄鱼MIF能加速胰岛素还原反应，具有TPOR活性，但MIF的催化反应受pH值和底物GSH的浓度影响较大；另外还从肝脾中分离培养了眼斑拟石首鱼的巨噬细胞，利用化学法测定了NO生成量，表明眼斑拟石首鱼的巨噬细胞能产生NO，添加了大黄鱼MIF融合蛋白后能诱导NO合成量的增加。生物学功能分析表明所获得的MIF重组蛋白具有生物学活性，研究结果为进一步开展鱼MIF的功能研究奠定了基础。

关键词：大黄鱼；眼斑拟石首鱼；天然免疫因子

Abstract

Fishes are in intimate contact with a rich microbial flora and exposed to millions of potential pathogens daily, and commercial cultures have faced increasing and appreciable economic damage in recent years. This thesis was in this research context of large yellow croaker genetic breeding for resistance to pathogen vibrio, and studied on cloning, expression and bioactivity analysis of immune related genes in an effort to lay the groundwork for understanding their molecular mechanism about bacteria-recognization as well as their roles in pathogenesis of vibriosis.

Mammalian macrophage migration inhibitory factor (MIF) is a multi-functional cytokine, which appears to be a critical controller in the pathogenesis of inflammatory disease. However, no information is available on MIF's comparative function in the pathogenesis of inflammatory infection of non-mammalian vertebrates; Peptidoglycan receptor is a hot-spot in molecular immunology research, one of which, pglyrp2, is a peptidoglycan-lytic amidase. Recently, the pglyrp2 homologue has been reported in zebra fish, where it exhibits both peptidoglycan-lytic amidase activity and broad-spectrum bactericidal activity, which is a newly-discovered feature in PGRP family. Therefore, this thesis studied on gene cloning and sequence analysis of pglyrp2 and MIF gene from two species of croakers, and then focused on large yellow croaker for mRNA expression, protein expression and bioactivity analysis of the two innate immune factors. The results are showed as follows:

1. cDNA cloning and gene cloning of MIFs of two species of croakers

This thesis first reported two MIF cDNA sequences which were cloned from the livers of *P.crocea* and *S.ocellatus* using homologue cloning technique and RACE approach, and also reported two MIF gene sequences which were cloned using muscle genomic DNA as template (Gene bank accession number of *P. crocea* MIF (LycMIF) and *S.ocellatus* MIF (SoMIF) were FJ415099 and FJ447489 respectively).

The full-length MIF cDNA of these two fish both included a 345 bp open

reading frame (ORF) which encoded a 115-aa protein; Predication of the signal peptide using the SignalP V3.0 software demonstrated that the two MIFs both lacked a signal peptide, suggesting that the two fish MIFs were secreted from cells by a non-classical, leaderless secretion pathway as mammalian MIFs; In addition, gene organization revealed that these two fish both contained 3 exons and 2 introns, and all exons, introns and aa sequences were highly similar between these two fish; Structure analysis showed that LycMIF contained a CALC motif which was typically found in Thiol-protein Oxidoreductases (TPORs) of the thioredoxin (Trx) family of proteins.

2. cDNA cloning and gene cloning of pglyrp2s of two species of croakers

Using the similar procedure above, cDNA and gene sequences of pglyrp2 of the two fish were obtained, the gene bank accession numbers of pglyrp2 cDNA sequences of the two fish were FJ385025 and FJ415100 while the gene bank accession numbers of pglyrp2 gene sequences of the two fish were FJ385024 and FJ415101 respectively.

The full-length pglyrp2 cDNAs of the two fish both included a 1446 bp ORF, which encoded a 482-aa protein. Predication of the signal peptide using the SignalP V3.0 software indicated that the two pglyrp2s both contained an N-terminal signal peptide, implying that the fish pglyrp2s were secretory proteins. In addition, sequence analysis revealed that the two fish pglyrp2 both contained 4 exons and 3 introns and they had highly similarity. As shown in the phylogenetic tree, fish pglyrp2 had close genetic relationship and belonged to the same monophyletic group as mammalian pglyrp2. Moreover, the pglyrp2 of *P. crocea* was predicted to have amidase activity because it had the required four Zn^{2+}-binding amino acids which were conserved in all amidase-active PGRPs.

3. Tissue distribution of MIF and pglyrp2 gene transcripts of *P. crocea*

Pglyrp2 and MIF gene transcripts were examined in tissues by RT-PCR and QRT-PCR analysis, including liver, gonad, gill, spleen, muscle, intestine, stomach, head kidney and brain. The results demonstrated that MIF mRNA was constitutively expressed in all selected tissues and was abundant in brain and liver, suggesting that fish MIF possibly has important biological functions; By contrast, pglyrp2 gene of *P. crocea* was strongly expressed in liver, weakly expressed in ovary, testis, intestine

and stomach, and undetected in other tissues, which displayed distinct tissue-specific expression pattern. In addition, maternally derived pglyrp2 mRNA displayed a high level in unfertilized eggs, followed by low expression throughout embryogenesis and yolk-sac larvae, implying that pglyrp2 might play an important role in defending the eggs.

4. The inducible MIF mRNA expression pattern following exposure to *V.harveyi*

The inducible mRNA expression pattern of MIF was observed in large yellow croaker following exposure to *V. harveyi* by qRT-PCR assay. As clinical observation and histological analysis demonstrated, the prominent features of the *V.harveyi*-infected large yellow croaker were the gross epidermal ulcer and internal tissue damage mainly to the liver, head kidney and intestine. In addition, there was a significant increase of expression of LycMIF gene in liver ($P<0.01$) and head kidney ($P<0.05$), but no significant difference was observed in gill and intestine. The present study represented the first description of the pathological profile of *V. harveyi*-infected large yellow croaker, and also provided the first direct evidence that fish MIF was implicated in pathogenesis of fish vibriosis.

5. The inducible pglyrp2 mRNA expression pattern

In order to explore the role of pglyrp2 in bacteria recognition, SOD activity assay was employed to analyze anti-oxidant defense response while QRT-PCR was used for examining the mRNA expression level of pglyrp2 of *P. crocea* in response to different bacteria, Gram-positive *Micrococcus lysoleikticus* (Sigma) and Gram-negative *Vibrio Harveyi* TS-628.

Although *V.harveyi* had caused vibriosis while *M. lysoleikticus* had caused no apparent ulceration, they induced both up-regulation of mRNA expression and subsequent increase in SOD activity, suggesting that pglyrp2 possibly had no specificity in bacteria recognition and possibly plays an important role in initiating downstream antimicrobial immune defense response.

6. Expression and purification of MIF and pglyrp2 proteins

The recombinant plasmid pET-LycMIF, pET-pglyrp2 and pET- pglyrp2 mature peptide were constructed according to the obtained MIF and pglyrp2 cDNA