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厦门大学

博士学位论文

拟穴青蟹两种性腺高表达抗菌肽Scygonadin和SCY2特性比较及其生殖免疫机制的研究

Comparison of two highly expressed antimicrobial peptides Scygonadin and SCY2 in Scylla paramamosain gonads and their mechanism of reproductive immunology

乔琨

指导教师：王克坚

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摘 要

拟穴青蟹(Scylla paramamosain，以下简称青蟹)是我国南方最重要的海水养殖蟹类品种。近些年来，随着青蟹养殖规模的不断扩大，各种病害频繁发生引起养殖青蟹大量死亡，严重影响了我国青蟹养殖业的发展。青蟹以先天性免疫为主，抗菌肽是先天性免疫系统中重要免疫因子，对抵抗病原微生物感染发挥关键作用。Scygonadin(SCY1)和SCY2是本实验室从雄性拟穴青蟹射精管中发现的两种新抗菌肽基因，前期研究已分别阐明了两种抗菌肽的基因组成与部分特性，并证明SCY1具有生殖免疫功能。青蟹半开放的生殖系统使得生殖道长期暴露于潜在病原微生物的环境中，因此研究该类抗菌肽与生殖免疫的关系对于揭示海水养殖环境下青蟹如何成功受精与保障繁育的机制具有重要意义。本论文较系统地研究了SCY1和SCY2基因在自然状态和不同月份青蟹中表达特性；以及细菌、病毒感染条件下这两种抗菌肽的表达模式；阐明了交配过程和眼柄摘除对SCY1和SCY2基因的诱导表达；并通过建立青蟹原代细胞培养方法，探讨了几种激素对青蟹体内和体外基因的诱导模式及启动子活性研究。该项研究为深入揭示SCY1和SCY2抗菌肽在青蟹生殖免疫中的作用奠定了科学基础。主要结果如下：

1. 自然状态下正常雄性及雌性青蟹各组织器官中SCY1和SCY2基因和蛋白的表达特性。利用荧光定量PCR检测正常雄、雌性青蟹SCY1和SCY2基因组织表达特性的结果显示，在雄、雌性青蟹体内，所检测的各器官和组织均有一定量的SCY1和SCY2基因表达。两个基因的表达模式较相似，其中射精管的表达量最高，其次为后射精管、阴茎，在雄蟹其他组织器官和雌蟹中有微弱的表达；射精管的表达量与其他组织器官中的表达量之间均存在显著差异($P<0.05$)。Western Blot和免疫荧光结果显示，雄性青蟹射精管中存在大量的SCY1和SCY2蛋白，其次是后射精管和阴茎，而其他组织器官中均未检测到这两种抗菌肽。

2. 全年不同月份SCY1和SCY2基因和蛋白的表达特性。荧光定量PCR检测了1月至12月份雄性拟穴青蟹SCY1和SCY2的基因表达。结果显示，两组体重的青蟹中SCY1和SCY2基因均在11月份和6月份表达量最高。Western Blot结果与荧光定量PCR的结果相一致，SCY1，SCY2蛋白在5，6月份和10，11月份有大量表达。比较发现，两种抗

菌肽的表达规律与青蟹的性成熟期一致，暗示SCY1和SCY2的表达模式与交配诱导相关。

3. 细菌感染条件下青蟹抗菌肽SCY1和SCY2的表达特性。利用荧光定量PCR，检测了溶藻弧菌感染后青蟹射精管中SCY1和SCY2的表达变化。在细菌感染3 h和6 h，SCY1被显著性地抑制表达(P<0.05)，12-96 h基因表达水平较对照组均无显著变化。SCY2在细菌感染3 h，6 h以及48 h均被显著抑制(P<0.05)，其他时间点基因表达水平与对照组无显著变化。

4. 抗菌肽SCY2基因抑制对WSSV病毒感染青蟹的影响。荧光定量PCR检测了WSSV感染雄性青蟹IE1基因各组织器官扩增情况，结果显示在雄性青蟹体内，鳃中IE1表达量最高，其次为心脏、脑神经节，胸神经团，其他组织有低量的表达，而血液和肝脏中均未检测到IE1基因。用病毒感染SCY2抑制后的青蟹，结果发现射精管中IE1表达量高于对照组，并且注射siRNA不会引起青蟹体内免疫指标显著性变化。

5. 交配至胚胎发育过程中青蟹SCY1和SCY2基因与蛋白的表达模式。通过荧光定量PCR检测了交配前后青蟹SCY1和SCY2在各性腺器官的表达特性。结果发现，SCY1与SCY2基因表达趋势较一致，在射精管中交配前SCY1和SCY2表达量高于交配后；在后射精管、阴茎和雌蟹纳精囊中，交配后基因表达量高于交配前。Western Blot结果显示，在射精管中交配前SCY1和SCY2蛋白表达量高于交配后。利用免疫荧光法在未交配的雌性青蟹阴道及纳精囊中均没有检测到SCY2蛋白，而在交配后的雌性青蟹阴道的几丁质层以内的填充物及纳精囊中精荚周围的内腔里出现SCY1和SCY2阳性。

进一步对胚胎中SCY1和SCY2基因和蛋白水平的检测发现，荧光定量PCR可以检测到青蟹胚胎和幼体中微量的SCY1和SCY2基因表达，但Western Blot、免疫荧光和免疫组化方法均检测不到胚胎中SCY1和SCY2蛋白分布。

6. 眼柄摘除后SCY1和SCY2基因的表达特性。利用荧光定量PCR检测了青蟹眼柄摘除后3 d和7 dSCY1和SCY2基因表达水平。结果发现，在眼柄摘除后3 d射精管与精巢中SCY1和SCY2基因的表达量均低于对照组，而在7 d表达量均高于对照组。Western Blot结果显示摘除眼柄后7 d的青蟹射精管中SCY1和SCY2蛋白含量明显高于对照组和其他时间点。

7. 青蟹血细胞原代培养及性腺细胞分离的方法。通过对青蟹血细胞原代培养条件

的摸索与优化，建立了血细胞原代培养的方法及体系。利用培养的青蟹原代血细胞进行了功能基因的检测，该细胞可以满足短期体外功能实验的需求。并探索了青蟹性腺细胞的分离方法，对体外培养的性腺细胞进行了RNAi试验。

8. 甲壳动物类固醇激素与青蟹抗菌肽SCY1和SCY2的表达关系。拟穴青蟹蜕皮激素体内诱导实验结果显示，在注射蜕皮激素后各时间点SCY1和SCY2基因表达量与对照组无显著性差异。对甲壳动物性类固醇激素(17β-雌二醇，孕酮及睾丸酮)进行的体外诱导试验，结果表明孕酮可以显著诱导SCY1和SCY2的基因表达，并且这种作用可被孕酮受体抑制剂所抑制。进一步比较了LPS与孕酮对青蟹射精管SCY1和SCY2的作用，发现LPS不能诱导这两种抗菌肽的表达，但是其受体TLR可以显著被诱导。

9. SCY2基因启动子活性与激素诱导相关。构建了青蟹SCY2-pGL启动子载体，通过对SCY2启动子活性的研究，发现孕激素能诱导SCY2启动子活性，但是LPS对SCY2启动子没有显著诱导，推测激素诱导该基因表达的途径可能是通过其上游调控序列中孕激素受体元件。

关键词：拟穴青蟹；Scygonadin；交配；激素；生殖免疫

Abstract

The mud crab, Scylla paramamosain is the most important marine breeding crab in northern China. In recent years, the farming of this species has grown rapidly. However, this economic species frequently suffered from outbreaks of disease which had led to decrease in production and severe economic loss. Antimicrobial peptides are major component of the innate immune defense system in S. paramamosain. They are important in host defense against all forms of microbial pathogens.

Scygonadin (SCY1) and SCY2 are important antimicrobial peptides which were ﬁrst isolated from the ejaculatory duct of S. paramamosain in our laboratory. Our previous studies have shown partial gene organization and expression patterns of these two genes, and confirm SCY1 is associated with the reproductive immunity of crabs. The semi-open reproductive system of crab results in the reproductive duct continuously exposed to an environment in which there might be potential pathogens causing diseases. Study on the relationship between antimicrobial peptide and reproductive immunity is meaningful in clarifying how to successfully fertilize under mariculture environment and protect breeding.

In order to clarify the expression characteristics and functions of these two genes, the tissue-specific distribution and seasonal changes of SCY1 and SCY2 were detected. Their expression patterns during Vibrio alginolyticus and WSSV in S. paramamosain were also determined. The expressions of SCY1 and SCY2 were studied following mating and eyestalk ablation. Furthermore, the impacts of hormones on SCY1 and SCY2, as well as promoter activity were performed by culturing the primary cell. Thus, this study provides the basis for further understanding of the reproductive immunity of S. paramamosain. The results were as follows:

1. mRNA and protein expression of SCY1 and SCY2. The mRNA expression of SCY1 and SCY2 were detected for all tissues of both male and female S. paramamosain. The highest expression level of these two genes was present in the ejaculatory duct, and a relatively low expression was also observed in post-ejaculatory duct and penis, while other tissues tested showed poor expression. SCY1 and SCY2 gene expression in the ejaculatory duct was signiﬁcantly higher than that in other tissues ($P<0.05$). The SCY1 and SCY2 protein expression pattern was studied using Western Blot and Immunofluorescence. The highest expression level was in the ejaculatory duct, and was also observed in post-ejaculatory duct and penis. These proteins were not detected other tissues.

2. Seasonal changes of SCY1 and SCY2 mRNA expression in the sexually mature crabs. Both genes had high levels of expression in June and November. High expression level of SCY1 and SCY2 protein appeared in May, June, October and November.The expression patterns of these two genes were consistent with sexual maturity of S. paramamosain.These results suggested that these two genes might be associated with the reproductive event.

3. Analysis of SCY1 and SCY2 gene transcription of S. paramamosain after challenge with V. alginolyticus. The results showed that SCY1 mRNA was significantly downregulated at 3 h and 6 h, while SCY2 mRNA was significantly downregulated at 3 h, 6 h and 48 h ($P<0.05$).There was no significant difference between bacteria and PBS challenged group during other time point.

4. Effect of WSSV infected S. paramamosain after SCY2 RNAi. The tissue distribution of IE1 from S. paramamosain challenged with WSSV was investigated. The highest expression level of IE1 was present in the gill, and a relatively high expression was observed in the heart, brain and thoracic ganglion mass. However, other tissues showed a relatively low expression. Expression of IE1 mRNA of WSSV infected S. paramamosain after SCY2 RNAi increased

relative to the control. There were no significant differences in RNAi on lysozyme activity and MDA in the serum of S. paramamosain.

5. SCY1 and SCY2 mRNA transcripts of pre- and post-mating S. paramamosain. The results showed that the expression level of the SCY1 and SCY2 genes in the ejaculatory duct was decreased after mating, but increased in the post-ejaculatory duct and penis in males, and in the spermatheca in females. The SCY1 and SCY2 protein level in pre- and post-mating crabs were studied using Western Blot and Immunofluorescence. Protein levels showed the same pattern as that of levels of gene expression. In female crabs, a positive signal was detected in spermatheca and vagina after mating. SCY1 and SCY2 gene expression in different developmental stages of S. paramamosain was also examined. Both embryo and larvae showed poor gene expression, and the protein level was not detectable.

6. Effect of eyestalk ablation on SCY1 and SCY2 gene expression of S. paramamosain. The results showed that SCY1 and SCY2 mRNA expression levels were downregulated at 3 d, but upregulated at 7 d. Protein levels significantly increased at 7 d.

7. Culture of S. paramamosain hemocytes. The optimized primary hemocyte of S. paramamosain was successfully cultured, and used for in vitro experiments. Isolation, culture, and function of germ cell from S. paramamosain were also analyzed.

8. The relationship between hormones and the expression of SCY1 and SCY2 in S. paramamosain. Induced expression of SCY1 and SCY2 genes in S. paramamosain following hormone was examined. There was no significant difference between the control and the 20-Hydroxyecdysone challenged group. In vitro induction of SCY1 and SCY2 expression by sex steroid on the ejaculatory duct of S. paramamosain was investigated. The results showed that SCY1 and SCY2 mRNA were significantly upregulated by progesterone, but not estradiol

17β or testosterone. In this study we compared the effect of LPS to hormones on the expression of the SCY1 and SCY2 genes. The results showed that LPS induced TLR, but had no effect on SCY1 and SCY2 expression.

9. The potential transcription factor binding sites and promoter activity of SCY1 and SCY2 DNA flanking from S. paramamosain. Progesterone stimulation increased SCY2 promoter activity, while LPS had no effect on SCY2 promoter activity.

Keywords: Scylla paramamosain; Scygonadin; Mating; Hormone; Reproductive Immunity

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