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鳌虾免疫相关基因的检出及丝氨酸蛋白酶抑制物基因分析

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摘要：通过对抑制性差减杂交和cDNA芯片技术分离的201个阳性克隆的测序，得到鳌虾(*Procambarus clarkii*)免疫相关基因48个，其中正向克隆中42个，反向克隆6个，除正向克隆中SNAP-25(synatosome-associated protein of 25 ku)已报道外，其余均为新基因，均在GenBank登录。正向克隆中的同源基因有微管蛋白、超氧化物歧化酶前体、丝氨酸蛋白酶抑制物Ⅰ、精氨酸激酶、70 kD伴侣蛋白同类物等。进一步用Dot Northern blot对克隆号PCI188进行鉴定，结果攻毒组的杂交信号是对照组的3.24倍，与cDNA芯片结果相符。用快速扩增cDNA末端技术扩增克隆号PCI188基因的5'端片段和3'端片段，全长共1 128 bp，编码的蛋白质有277个氨基酸，分子量为30.27 kD。与GenBank序列号X79512软尾太平刺蛄(*P. leniusculus*)的蛋白酶抑制物Ⅰ(为丝氨酸蛋白酶抑制物)的基因同源性为58.7%，氨基酸同源性为69.7%。该蛋白质有5个重复的结构域，与丝氨酸蛋白酶抑制物Kazal家族的结构域相似。

关键词：鳌虾；cDNA芯片技术；免疫相关基因；丝氨酸蛋白酶抑制物

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对虾白斑综合症病毒(white spot syndrome virus，WSSV)给全球对虾养殖业带来了巨大的经济损失，因而吸引了众多学者从事该病毒的研究。由于没有WSSV体外增殖的细胞，对虾在实验室条件下又不易暂养，且带毒多，不利于进一步实验研究，动物模型的研究迫在眉睫。本实验室率先开展了WSSV克氏原鳌虾(*Procambarus clarkii*，以下简称鳌虾)动物模型的研究[1-2]，鳌虾天然不带毒、易于饲养、对WSSV敏感，现已成为研究WSSV的良好试验动物而得到广泛地应用。

对虾属于甲壳类，没有特异性的免疫系统，其天然免疫系统的报道还不系统，相关研究如酚氧化酶原激活系统(prophenoloxidase activating system，proPO系统)也多以鳌虾作为研究对象[3-4]。

本实验室利用抑制性差减杂交结合cDNA芯片技术，依据已报道的WSSV全基因序列，鉴定了其中的鳌虾免疫相关基因，并在GenBank上登录了一批新基因，为甲壳类免疫系统的后续研究打下了基础。鳌虾丝氨酸蛋白酶抑制物基因的检出，充实了甲壳动物酚氧化酶原激活系统的调控理论。

1 材料与方法

1.1 材料

1.1.1 鳌虾 购自南京某农贸市场，水温22 ℃，实验室饲养1周以上，健康存活。

1.1.2 白斑综合症病毒青岛株(WSSV) 经鳌虾传18代，本室保存。

1.1.3 总RNA 采用TriPure分离液(Roche公司产品，Cat. No. 1667165)，mRNA的提取采用Oligotex Direct mRNA Mini Kit(QIAGEN公司产品，Cat. No. 72022)提取。

1.1.4 抑制性差减杂交 采用PCR-Select™ cDNA Subtraction Kit(CLONTECH产品，Cat. No. K1804-1)技术。

1.1.5 快速扩增cDNA末端技术(rapid amplification of cDNA end，RACE) 采用SMARTTMRACE cDNA Amplification Kit(CLONTECH产品，Cat. No. K1811-1)。

1.2 方法

1.2.1 抑制性差减杂交文库的构建 病毒的提取

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Isolation and identification of pathogenetic *Vibrio harveyi* from estuary cod *Epinephelus coioides*

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Abstract: An outbreak of serious mortality among the cultured estuary cods, *Epinephelus coioides*, characterized by an ulcer on the surface of moribund estuary cods' body, occurred in Guangdong in April, 2003. One strain EcGY020401 was isolated from the liver of diseased estuary cod. Traditional physiological and biochemical methods, together with API system were applied in the bacterial classification. This strain was incubated with tryptic soy agar (TSA) supplemented with 0.85% NaCl and thiosulfate citrate bile sucrose agar(TCBS). As a result, it is negative and shows yellow in TCBS. There is no ornithine ecarboxylase, arginine dihydrolase, Lysine decarboxylase, Urease and so on in the strain. There is cellobiose, sorbitol, glucose and sucrose in the strain. The strain was confirmed to be the pathogen causing the disease in *E. coioides* through artificial infection test. The LD_{50} value of the strain for estuary cod was 2.7×10^6 CFU/g estuary cod body weight (using Reed-Muench method). The bacteria was reisolated from liver after bacterial challenge. To determine the status of strain EcGY020401, the strain's 16S rRNA sequence was analyzed by Vectlr NTI suit 6.0 software. The results showed the reisolated strain is the same species as strain EcGY020401. The results also showed that 16S rRNA sequence length of strain EcGY020401 is 1 379 bp. A system evolution tree was set up, showing strain EcGY020401 is the nearest to *V. harveyi*. Concolusionly, strain EcGY020401 belongs to *V. harveyi*. This study demomstrated that strain EcGY020401 does not radiate, which is not agreeable with reported resources. In addition, this study showed that strain EcGY020401 does not grow when salinity is 0 while it grows very well when salinity changes from 15 to 100. In conclusion, the strain is salinity-trophile. The test of sensitivities of strain EcGY020401 to 20 kinds of antibiotics revealed that the pathogen is sensitive to drugs such as Rifampixcin(RA), tetracycline(TE) and Ceftriaxone(CRO).

Key words: *Epinephelus coioides*; *Vibrio harveyi*; 16S rRNA

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及接种参照朱建中等[2]方法进行。对照组鳌虾每尾用0.01 mol/L PBS(pH 7.4)腹节肌肉注射0.1 mL。接毒3天后,用一次性注射器由鳌虾心脏采取血淋巴,以接种WSSV的鳌虾为试验组,注射PBS的鳌虾为对照组,分别抽取它们的血淋巴,提取总RNA和mRNA。根据抑制性差减杂交试剂盒的操作程序,通过正向和反向差减杂交,建立鳌虾接种WSSV后上扬基因和抑制基因文库。

1.2.2　cDNA芯片制备及检测　cDNA芯片的制备及检测由上海中科开瑞生物芯片科技股份有限公司完成,取2倍以上差异表达基因为阳性。

1.2.3　测序分析　选择芯片技术鉴定的阳性克隆,接种培养基后,送上海中科开瑞生物芯片科技股份有限公司进行测序,测序结果分别用Blastn(Basic Local Alignment Search Tool)与核酸数据库GenBank进行"核苷酸序列关于核酸序列数据库的同源性检索",取最高同源性检索结果。

1.2.4　Dot Northern blot验证　Dot Northern blot验证参照《现代分子生物学实验技术》[5]进行。用BIO-RAD的凝胶成像系统对杂交信号进行扫描,并用Quantity one分析软件进行灰度分析。

1.2.5　鳌虾丝氨酸蛋白酶抑制物基因分析　采用SMARTTMRACE cDNA Amplification Kit,按说明书操作。根据克隆号PCI 188的基因序列设计3'RACE和5'RACE引物如下:

3'RACE引物:5'-CGC GGC TCG CAA CAA CCC ACA ACT GC-3'

5'RACE引物:5'-GAT TTT GCG GCC TAC ATT CGC CTT GG-3'

将PCR产物接入pMD18-T载体测序。

2　结果

本研究构建的cDNA芯片含克隆1 514个,鉴定的阳性克隆共计281个。完成了201个克隆的测序工作,所得结果在GenBank中进行了同源性比较,除去WSSV表达基因30个,得到鳌虾的免疫相关基因48个,其中接种WSSV后表达量上升的有42个,下降的有6个,在GenBank登录基因序列47个,具体结果见表1。

克隆号PCI 188及鳌虾丝氨酸蛋白酶抑制物的Dot Northern blot鉴定结果见图1,阳性对照信号明显,阴性对照无信号,实验组中的信号是对照组的3.24倍,与芯片的3.03倍相符。

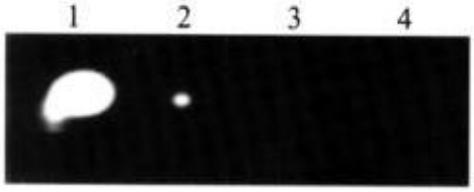

图1　克隆号PCI188RNA斑点杂交检测结果

1.阳性对照,2.试验组,3.对照组,4.阴性对照

Fig.1　Dot Northern blot of experiment group and control group with PCI188 as probe

1, Positive control. 2, Experiment group. 3, Control group. 4, Negative group

鳌虾丝氨酸蛋白酶抑制物的cDNA 3'RACE扩增得到一条600 bp的条带,如图2所示。5'RACE也得到一条约800 bp的条带,如图3所示。

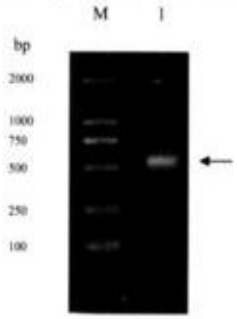

图2　鳌虾丝氨酸蛋白酶抑制物cDNA的3'RACE电泳图(箭头示目的条带)

Fig.2　Electrophoresis of the 3'RACE of serine protease inhibitor cDNA of crayfish (Arrow means the amplified fragment)

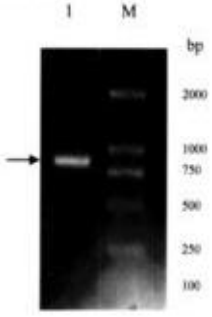

图3　鳌虾丝氨酸蛋白酶抑制物cDNA的5'RACE电泳图(箭头示目的条带)

Fig.3　Electrophoresis of the 5'RACE of serine protease inhibitor cDNA of crayfish (Arrow means the amplified fragment)

表1 螯虾免疫相关基因

Table 1 Immune gene of crayfish *Procambarus clarkii*

克隆号 Clone no.	芯片中的差异倍数 Ratio(experiment group/control group)	检测重复次数 Detection times	GenBank 登录号 GenBank accession number	备注 Note
PCI 039	2.82	2	AY266981	上扬基因 Upregulated gene
PCI 015	2.55	4	AY266984	上扬基因 Upregulated gene
PCI 025	2.93	2	CD644784	上扬基因 Upregulated gene
PCI 032	8.46	3	AY266982	上扬基因 Upregulated gene
PCI 011	2.41	2	AY266985	上扬基因 Upregulated gene
PCI 060	5.57	1	AY266988	上扬基因 Upregulated gene
PCI 054	2.64	2	AY266986	上扬基因 Upregulated gene
PCI 049	2.74	2	AY266983	上扬基因 Upregulated gene
PCI 109	12.23	1	CB832305	上扬基因 Upregulated gene
PCI 036	3.21	2	CB832308	上扬基因 Upregulated gene
PCI 093	3.02	2	CB832306	上扬基因 Upregulated gene
PCI 033	2.84	2	CB832304	上扬基因 Upregulated gene
PCI 048	2.22	2	CB832307	上扬基因 Upregulated gene
PCI 232	2.09	1	CD644781	上扬基因 Upregulated gene
PCI 291	2.04	2	CD644792	上扬基因 Upregulated gene
PCI 189	2.06	2	CD644776	上扬基因 Upregulated gene
PCI 195①	2.23	1		上扬基因 Upregulated gene
PCI 295②	3.46	2	CD670485	上扬基因 Upregulated gene
PCI 164	2.63	2	CD644772	上扬基因 Upregulated gene
PCI 184	2.23	1	CD644774	上扬基因 Upregulated gene
PCI 188②	3.03	1	CD644775	上扬基因 Upregulated gene
PCI 271	2.05	2	CD644785	上扬基因 Upregulated gene
PCI 173	3.15	2	CD644773	上扬基因 Upregulated gene
PCI 210	2.14	2	CD644777	上扬基因 Upregulated gene
PCI 220	2.53	1	CD644778	上扬基因 Upregulated gene
PCI 230	2.15	1	CD644780	上扬基因 Upregulated gene
PCI 290④	2.35	1	CD644791	上扬基因 Upregulated gene
PCI 096	3.58	1	CD644793	上扬基因 Upregulated gene
PCI 225	3.94	2	CD644779	上扬基因 Upregulated gene
PCI 240	2.65	2	CD644782	上扬基因 Upregulated gene
PCI 243	2.26	1	CD644783	上扬基因 Upregulated gene
PCI 277	4.33	2	CD644787	上扬基因 Upregulated gene
PCI 116③	2.84	1	CD670482	上扬基因 Upregulated gene
PCI 162	2.45	1	CD644771	上扬基因 Upregulated gene
PCI 246④	2.24	1	CD670484	上扬基因 Upregulated gene
PCI 273	4.05	2	CD644786	上扬基因 Upregulated gene
PCI 285	2.53	1	CD644788	上扬基因 Upregulated gene
PCI 287	2.36	2	CD644790	上扬基因 Upregulated gene
PCI 286	2.14	1	CD644789	上扬基因 Upregulated gene
PCI 248	2.35	1	CD670481	上扬基因 Upregulated gene
PCI 156	2.69	1	CD670483	上扬基因 Upregulated gene
PCI 072	2.21	1	CD670486	上扬基因 Upregulated gene
PCI 073	0.28	2	AY266987	抑制基因 Downregulated gene
PCI 150	0.44	2	CD644770	抑制基因 Downregulated gene
PCI 111	0.47	2	CD644768	抑制基因 Downregulated gene
PCI 112	0.33	2	CD644769	抑制基因 Downregulated gene
PCI 146	0.44	4	CD573613	抑制基因 Downregulated gene
PCI 159	0.48	5	CD573611	抑制基因 Downregulated gene

表 1 注:①克隆号 PCI 195 与 GenBank 登录号 AB063358 鳌虾突出小体相关蛋白 SNAP25mRNA 部分序列 100% 同源;②克隆号 PCI 295 与 GenBank 登录号 HAU68764 美国龙虾(*Homarus americanus*)的 α－Ⅲ微管蛋白 mRNA 部分片段 87% 同源;③克隆号 PCI 188 与 GenBank 登录号 X79512 软尾太平刺蛄(*Pacifastacus leniusculus*)的蛋白酶抑制物Ⅰ mRNA 的部分片段 92% 同源;④克隆号 PCI 290 与 GenBank 登录号 AF122900 软尾太平刺蛄胞外超氧化物歧化酶前体 mRNA 的部分片段 86% 同源;⑤克隆号 PCI 116 与 GenBank 登录号 AF167313 岸蟹(*Carcinus maenas*)精氨酸激酶 mRNA 的部分片段 90% 同源;⑥克隆号 PCI 246 与 GenBank 登录号 AB016836 家蚕(*Bombyx mori*)的 70 kD 热休克蛋白同类物 mRNA 的部分片段 78% 同源。

Note of table 1: ①PCI 195 sequence showed 100% identity with that of the crayfish (*Procambarus clarkii*) SNAP25 mRNA for synaptosome-associated protein of 25 kD (GenBank accession number AB063358); ②PCI 295 sequence showed 87% identity with that of the American lobster (*Homarus americanus*) alpha－Ⅲ tubulin mRNA (GenBAnk accession number HAU68764); ③PCI 188 sequence showed 92% identity with that of signal crayfish (*Pacifastacus leniusculus*) mRNA for protease inhibitor Ⅰ (GenBank accession number X79512); ④PCI 290 sequence showed 86% identity with that of signal crayfish (*Pacifastacus leniusculus*) mRNA for extracellular superoxide dismutase precursor (GenBank accession number AF122900); ⑤PCI 116 sequence showed 90% identity with that of green crab (*Carcinus maenas*) arginine kinase mRNA (GenBank accession number AF167313); ⑥PCI 246 sequence showed 78% identity with that of domestic silkworm (*Bombyx mori*) mRNA for heat shock 70 kD protein congate (GenBank accession number AB016836).

根据 3' RACE 和 5' RACE 的测序结果拼接得鳌虾丝氨酸蛋白酶抑制物全长基因如图 4 所示。

基因全长 1 128 bp,其阅读框从 75 位到 908 位(图 4 阴影部分),GenBank 登录号为 AY351957,编码的蛋白质氨基酸序列如图 5 所示:

该蛋白质共包含 277 个氨基酸,分子量为 30.27 kD。DNAstar 软件分析表明其与 GenBank 序列号 X79512 软尾太平刺蛄(*P. leniusculus*)的蛋白酶抑制物Ⅰ的基因同源性为 58.7%,氨基酸同源性为 69.7%。进一步在 NCBI 中搜索其保守的结构域,发现它有 5 个 Kazal 型丝氨酸蛋白酶抑制物的保守结构域(图 5 阴影部分),分别位于 22～65 位氨基酸;73～118 位氨基酸;123～169 位氨基酸;174～220 位氨基酸;225～271 位氨基酸。同源的软尾太平刺蛄蛋白酶抑制物Ⅰ有 4 个类似结构域,分子量为 23 kD。因此,本研究发现的 GenBank 登录号为 AY351957 的基因为一新的丝氨酸蛋白酶抑制物基因。

AGTCGCGCTACGGCCGTCAGGTTGAAGCACAGTCACGGAGGGAGAGAGAGGTTGTGCTCTGGAGAC
TTGTGGAGATGCTGCGTTTACTGATCTGGGCCACAACCTTGTTGGTGGTCGGGGCTTCCACTCAGACC
AGGTGCCCAAGTGTGTGCCCATTCATCTTCCGACAAGTATGTGGCTCCGACAGCAAGTCGTATGCCAA
CGACTGCCTGCTCAATGTTGCCATTTGCAACAACCCCAACCTGAAGAAGTTGCATGATGGCCCCTGCA
GTGGCGGGTCAAAACCTCGGTGTCCCACTGTTTGTACCTTGGAGTACAAGCCGGTGTGTGGGACGGA
CGGCAAGACCTACAGCAACCGGTGCGCCCTCGAGGTCGAAGCCTGCAACAATCCTCAACTCAAGCTG
AGAATCGCCTACGAAGGCGAATGTAGGCATAAAAATCCGTGTCCTAAGGCTTGTACTCTGCAATATGAC
CCTGTGTGTGGAACAGATGGCAAGACTTACAGCAACTTGTGTGACCTAGAAGTCGAGGCCTGCAATA
ACCCGCAACTGAATCTGAAAGTAGCGTACAAAGGCGAATGTAGGCCGCAAAATCAGTGTAACAGCGT
TTGTCCTCAGATCTACCAGCCTGTGTGTGGGACAGACGGCAAGACGTACAGCAACCAGTGTACACTG
GACGTCGCGGCCTGCAACAACCCACAACTGCATCTTAGAACAGCCTACCAAGGCGAATGTAGGACGT
CCAATCAGTGTGGAAGCTTCTGTACACTACAGTACGATCCTGTGTGTGGGACTGACGGCAAGGACTACA
GCAACTCTTGCTTCCTAGGGATAGCTGCCTGCAGAAACCCTGGCTTGAATTTGAAGATCGCCTATAAA
GGCAGATGTAATTCTCGGCAGCTAAGTTAAAGAAGACTTGTGGACAAAGAGAATCTTCACTAAGGATC
TATATGGCAGAAAATCCCTAAATCTTGCTGTTGGCGGCTTTTCATGTTTTTCAAAGAGTTGACGCCGTC
GATTGAAGAGGCTATATGGAGGTATGGCGTCTATGGTGAACATTAGTAATGATCTGAATTGTGCTTGTAT
ATTGTGATAATGGAACCCTTCCTAATAAAGTTGCTAATAATGTAAAAAAAAAAAAAAAA

图 4　鳌虾丝氨酸蛋白酶抑制物 cDNA 的全长序列(阴影部分为阅读框)

Fig. 4　Complete sequence of cDNA of serine protease inhibitor of crayfish (Shadow means ORF)

MLRLLIWATTLLVVGASTQTRCPSVCPFIFRQVCGSDSKSYANDCLLNVAICNNPNLKKLHDGPCSGGSKP
RCPTVCTLEYKPVCGTDGKTYSNRCALEVEACNNPQLKLRIAYEGECRHKNPCPKACTLQYDPVCGTDG
KTYSNLCDLEVEACNNPQLNLKVAYKGECRPQNQCNSVCPQIYQPVCGTDGKTYSNQCTLDVAACNNPQ
LHLRTAYQGECRTSNQCGSFCTLQYDPVCGTDGKDYSNSCFLGIAACRNPGLNLKIAYKGRCNSRQLS

图 5　鳌虾丝氨酸蛋白酶抑制物的氨基酸序列(阴影部分为保守结构域)

Fig. 5　Amino acid sequence of serine protease inhibitor of crayfish (Shadow means conserved domain)

3 讨论

根据 Carlisle 等[6]和 Zhao 等[7]报道，cDNA 芯片筛选的2倍以上差异表达基因经 Northern blot 鉴定，其中约80%以上为真正的差异表达基因。Yang 等[8]用 Northern blot 鉴定 cDNA 芯片结果，11 个4倍以上差异表达克隆全部阳性；14 个3倍以上差异表达克隆中有13个被证实；而21个2倍以上差异表达克隆仍有17个被证实。可以看出 cDNA 芯片与 Northern blot 鉴定之间具有较高的符合率。本实验选用的克隆 PCI188 的 cDNA 芯片检测差异为3.03倍，经 Dot northern blot 验证为阳性，实验组信号是对照组的3.24倍，与基因芯片的检测结果相符，为后续开展全长基因分析提供了保障。

甲壳动物虽然没有特异免疫系统，其天然免疫系统也有细胞免疫和体液免疫之分[4,9-11]。但对其免疫系统还缺乏系统的研究，特别是在分子水平。因此对于本研究检出的大多数基因，在 GenBank 中都没有查到同源序列，属于新基因，但为进一步研究打下了基础。对检索到的几个有同源基因的功能分析见表1。

PCI 246，GenBank 登录号 CD670484，全长 352 bp，上扬基因，其中 239 bp 与家蚕（*Bombyx mori*）的 70 kD 热休克蛋白同类物 mRNA（GenBank 登录号 AB016836）的相应片段78%同源。已知热休克蛋白（HSP）是各种有机体在遭受应激刺激后诱导产生的一组应激蛋白，主要分为 HSP90（83～90 kD），HSP70（66～78 kD），HSP60 及小 HSP（15～39 kD）等4个家族。HSP70 是 HSP 中最保守和最主要的一类，在大多数生物中含量最多，在细胞应激后生成最为显著，对细胞损伤具有保护作用[12-14]。其生物学功能主要有：1）分子伴侣作用：保护新合成的蛋白分子的正确构型。2）提高细胞对应激原的耐受性。另外 HSP70 还参与抗原的加工、提呈等。对虾在接受弱毒刺激之后，再以强毒攻之，其存活率明显上升[15]，该实验现象与 HSP70 的功能正好吻合，因此，可以肯定 HSP70 在对虾的免疫保护中发挥了主要的功能。本实验对该基因的检出，进一步证实 HSP70 在甲壳动物的免疫防御中发挥积极作用。

PCI 295，GenBank 登录号 CD670485，全长 262 bp，上扬基因，其中 259 bp 与美国龙虾（*Homarus americanus*）的 α－Ⅲ微管蛋白 mRNA（GenBank 登录号 HAU68764）的相应片段87%同源。微管蛋白在真核生物细胞中普遍存在，是所有微管结构的主要组成，作为细胞骨架的重要成分，微管不仅在维持细胞形态、保持细胞内部结构的有序性中起重要作用，而且与细胞内的物质运输、细胞运动、细胞分化发育以及细胞分裂繁殖等生命活动密切相关。PCI 195，SNAP－25（synatosome－associated protein of 25 ku）是细胞分泌过程中介导小泡定向和融合的关键蛋白[16]。微管蛋白和 SNAP－25 都与细胞的活动相关，其表达量的上升可能与鳌虾经受刺激以后，体内各种信号传递活动增加有关。

PCI 290，GenBank 登录号 CD644791，全长 189 bp，上扬基因，其中 104 bp 与软尾太平刺鲀（*P. leniusculus*）胞外超氧化物歧化酶前体 mRNA（GenBank 登录号 AF122900）的相应片段86%同源。超氧化物歧化酶是含有铜、锌、锰和铁的金属酶，广泛存在于生物体的各种组织中，是唯一能够特异清除自由基 O_2^- 的抗氧化酶，自由基 O_2^- 可对机体产生伤害，因此超氧化物歧化酶表达量的增高也是机体在应激状态下的一种免疫保护功能。

PCI 116，GenBank 登录号 CD670482，全长 380 bp，上扬基因，其中 158 bp 与岸蟹（*Carcinus maenas*）的精氨酸激酶 mRNA（GenBank 登录号 AF167313）的相应片段90%同源。精氨酸激酶是属于磷酸激酶的一种，分布范围最广，包括脊椎动物、无脊椎动物、半索动物和被囊动物，是磷酸激酶中最古老的一个[17]。磷酸激酶的主要功能是产生 ATP，提供机体活动所需要的能量。机体被病毒侵袭之后，会产生各种应激反应，以抵御病害的入侵，保护自身的稳定，精氨酸激酶表达量的增加也反应了实验鳌虾的一种现实状态，急需能量，积极防御。

酚氧化酶原激活系统的激活过程目前还不是十分清楚，但可以肯定它包含一系列的丝氨酸蛋白酶活化过程[18-19]，其最终一步反应为酚氧化酶原通过一种丝氨酸蛋白酶（酚氧化酶原激活酶 ppA）的水解作用而激活[20]。而参与 proPO 系统的每一种蛋白酶都会受到一种或多种蛋白酶抑制物的调节[21]。因此，本研究关于鳌虾丝氨酸蛋白酶抑制物基因的发现有助于对甲壳动物酚氧化酶原系统的认识。

鳌虾在 WSSV 感染之后，蛋白酶抑制物活性升高，将导致酚氧化酶原激活酶活性受到抑制，从而使其重要的免疫系统－酚氧化酶原系统的活性下降，这正好验证了 Roux 等[22]的推断，即南美蓝对虾（*P. stylirostris*）在接种 WSSV 以后，酚氧化酶原

(proPO)基因在感染的最初 8 个小时呈上升趋势,而在随后的 16 h 和 32 h 则出现下降现象,而其丝氨酸蛋白酶的定量 PCR 分析表明,在监测的感染后 32 h 中,表达量呈上升趋势,推测此时对虾体内的蛋白酶抑制物的表达水平可能上升。这与本实验在鳌虾中观察到的情况相符。此丝氨酸蛋白酶抑制物在鳌虾酚氧化酶原系统中的具体作用还有待深入研究。

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Detection of immune associated genes and analysis of a new serine proteinase inhibitor gene in crayfish *Procambarus clarkii*

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Abstract: White spot syndrome virus (WSSV) is a major pathogen in cultured penaeid shrimp. Infection of penaeid shrimp by WSSV can result in mortality of up to 90% to 100% within 3 to 7 d. But crustacean host defense mechanisms and particularly host viral defense are relatively poorly understood. Health management requires an improved understanding of the molecular response of crustaceans to pathogens. Crayfish (*Procambarus clarkii*) which had been developed as an animal model to culture WSSV in our lab was used in this experiment. Combining suppression subtractive hybridization (SSH) and cDNA chip, the immune genes of crayfish were studied and 201 clones including 184 forward subtracted clones and 17 reverse subtracted clones constructed by SSH and cDNA chip were sequenced using Pinpoint-T vector sequence as primer. The sequences were aligned with GenBank using the BLASTn program. Fourty-eight immune genes of crayfish were detected, of which 42 were upregulated genes and the other 6 were downregulated genes. All of them were new genes except the SNAP-25 gene. Five upregulated genes had homologous genes in the GenBank. Their sequences showed 87%, 92%, 86%, 90% and 78% identity with those in American lobster (*Homarus americanus*) alpha-Ⅲ tubulin mRNA (GenBank accession number HAU68764), signal crayfish (*Pacifastacus leniusculus*) mRNA for protease inhibitor Ⅰ (GenBank accession number X79512), signal crayfish mRNA for extracellular superoxide dismutase precursor (GenBank accession number AF122900), green crab (*Carcinus maenas*) arginine kinase mRNA (GenBank accession number AF167313) and domestic silkworm (*Bombyx mori*) mRNA for heat shork 70 kD protein cognate (GenBank accession number AB016836), respectively. Totally 47 GenBank accession numbers were assigned. The SSH clones PCI188, which was similar with signal crayfish mRNA for protease inhibitor Ⅰ, was identified by Dot Northern blot. Analyzed by Quantity One software, the density was 3.24 times higher in the experiment group than that in the control group. This result was coincident with that got from cDNA chip. According to the sequence of PCI188, two primers was designed. And the 5' end and 3' end of the cDNA were amplified respectively. A 1 128 bp fragment, which encodes a signal sequence and a mature protein of 277 amino acids with a predicted molecular mass of 30.27 kD, was obtained. The nucleotide identity between this protein and protease inhibitor Ⅰ of *P. leniusculus* is 58.7%. And the amino acids identity between them is 69.7%. The amino acid sequence of this protein consists of five repeated stretches, indicating that the protein has five domains. The domains have significant sequence similarities to the serine protease inhibitors of Kazal family. Detection of serine protease inhibitor in the upregulated genes confirmed the hypothesis brought forward by Roux that proPO expression was not upregulated in the same virus-infected shrimp led to the speculation that WSSV infection probably activated the expression of a protease inhibitor(s) that blocked the activity of serine protease or the activity of the proPO.

Key words: crayfish; cDNA chip; immune genes; serine protease inhibitor

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