
Warangkana Narksen¹, Sittiruk Roytrakul², Wigrom Rungsins⁢³, Amornrat Promboon¹, Sununta Ratanapo¹

ABSTRACT

Androgenic gland (AG) in higher crustacean is responsible for the development of male characteristics. Implantation of the whole androgenic gland into giant freshwater prawn (*Macrobrachium rosenbergii* de Man) led to transformation of female recipient into male. The purposes of this research were to construct and study cDNA library from androgenic gland of the giant freshwater prawn. The 766 5’ ESTs from cDNA library of *M. rosenbergii* androgenic gland were established and categorized into seven categories on the basis of general function. The SMART domain search tool predicted the existence of a domain between residues 66-105 of *Mr-AGs* clone, which was similar to insulin growth factor-binding protein and to insulin/insulin-like growth factor/relaxin family. This is the domain similar to *Cq-IAG*, three AGHs in isopods. In tissue-specific study, RT-PCR analysis indicated that *Mr-AGs* was specific to the male prawn and *Mr-AGs* mRNA was mainly observed in AG and partially in vas deferens. The *Mr-AGs* is proposed as a novel gene and a candidate gene for androgenic gland hormone.

Keywords: Androgenic gland, Androgenic gland hormone, *Macrobrachium rosenbergii*, cDNA library

e-mail address: fscisnr@ku.ac.th, g4764165@ku.ac.th

¹ Department of Biochemistry, Faculty of Science, Kasetsart University
² Genome Institute, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency
³ Department of Zoology, Faculty of Science, Kasetsart University
INTRODUCTION

Giant freshwater prawn (*Macrobrachium rosenbergii* de Man) is an economic prawn of Thailand. Male giant freshwater prawns grow faster and reach higher weights at harvest than females since females use considerable energy in egg production. It has thus become obvious that an efficient biotechnology for producing all-male prawn populations is required, especially in countries in which economically valuable crustaceans constitute an important source of income.

In crustaceans, male sexual characteristics are induced by a peptide hormone called androgenic gland hormone (AGH) which is produced by the male-specific androgenic gland (AG). AG removal from immature giant freshwater prawn males resulted in sex reversal, with complete female differentiation. Similarly, androgenic gland implantations into immature females led to the development of the male reproductive system (Nagamine et al., 1980a and 1980b). To transform these female to male prawns, high production of AGH and its injection into female prawns would be a more practical way to increase the prawn production than grafting of AG. This present work attempts to identify AGH gene from a cDNA library of giant freshwater prawn’s AG.

METHODOLOGY

Prawn samples

Adult male and female giant freshwater prawns (*M. rosenbergii*) were purchased from a commercial market in Bangkok, Thailand.

Isolation of total RNA

Total RNA was extracted from AGs of *M. rosenbergii* using TRizol reagent (Gibco, USA).

Isolation of messenger RNA

Messenger RNA was isolated from the total RNA of *M. rosenbergii* androgenic glands using NucleoTrap mRNA Purification Kits (MACHEREY-NAGEL, Germany).

First strand cDNA synthesis

The first strand cDNAs for tissue-specific analysis were synthesized using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Germany).

cDNA library construction

cDNA library of *M. rosenbergii* AGs was constructed by the SMART cDNA library construction kit (Clontech, USA) according to the manufacturer’s protocol. The packaging reaction was performed by using Packagene Lambda DNA packaging system (Promega, USA). Plaques from an unamplified library were converted to colonies using *E. coli* BM25.8. To determine the size of the inserted cDNA from library, PCR was performed directly using the randomly picked clones as template by amplification with primers, SP6 (5’-CGATTTAGGTGACACTATAG-3’) and T7 (5’-
TAATACGACTCAGTAGG-3'). The PCR reaction was performed using Real Taq DNA polymerase kit (RBC, Taiwan). The mixture was conducted in 5 μl final volume. The final concentration of each component was 1X Real Taq PCR buffer with MgCl₂, 0.1 μM dNTP mix, 0.2 μM each SP6 and T7 primers, 1.25 U Real Taq DNA polymerase. PCR reaction was performed according to the following protocol: preheated at 94 °C for 3 min, followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min, followed by final extension at 72 °C for 7 min. The PCR products were separated on a 1.2% agarose gel in 1X TAE buffer and stained in 0.5 μg/ml ethidium bromide solution.

Sequencing of cDNA from library

One thousand recombinant clones containing products of expected sizes from M. rosenbergii AG cDNA library were propagated and purified using NucleoSpin Plasmid Extraction Kit (MACHEREY - NAGEL, Germany). Sequencing from 5' end was applied to 960 independent colonies by Dr. Kazuei Mita, Insect Genome Research Unit, National Institute of Agrobiological Science, Tsukuba, Ibaraki JAPAN. An average length of nucleotide sequences of the 776 readings was about 500 bp. After removal of vector-based sequences, 766 readings (98.7%) were analyzed further.

DNA Sequence Analysis

Homologous sequences were sought using BlastX and the cutoff was set at E-value $\leq 10^{-4}$. ESTs that matched known or predicted genes were categorized into different groups according to functions. The nucleotide sequences of no significant similarity were translated and calculated the theoretical molecular weight using the ExPASy Proteomics Server (http://www.expasy.org/tools/pi_tool.html). The deduced amino acid sequence was further considered by SMART (http://smart.embl-heidelberg.de/smart). The deduced amino acid sequences of unknown sequence were compared with the Cherax quadricarinatus insulin-like androgenic gland factor (Cq-IAG) and three pro-AGHs known in isopods (Manor et al., 2007.; Ohira et al., 2003; Okuno et al., 1997) by ClustalW.

Tissue-specific analysis by qualitative RT-PCR

Total RNA from AGs, testis, vas deferens and hepatopancreas of male M. rosenbergii and from ovary and hepatopancreas of female M. rosenbergii were prepared. After RT-PCR, the first strand cDNAs from various tissues were used as templates in PCR reaction. Each amplification was primed by a pair of the Mr-AGs – specific primer Mr-AGs-F (5'-TTCACCTGAACAAGTCCCATC-3') and Mr-AGs-R (5'-TGTCGCAGACCTAAAGGAAGA-3') and an actin cDNA fragment was amplified with two specific primers: actin-F (5'-CCCAGAGCAAGAGAGGTA-3') and actin-R (5'-GCGTATCCCTCAGATGGG-3') as control. PCR reaction was performed by using PCR master mix kit (Fermentas, Germany) according to the following protocol: preheated at 95 °C for 2 min, followed by 25 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72
C for 30 s, followed by a final extension at 72 °C for 7 min. The PCR products were separated on a 2% agarose gel in 1X TAE buffer and stained in 0.5 μg/ml ethidium bromide solution.

DNA sequencing

The sequence analysis was performed at Macrogen DNA sequencing service (Korea) by Applied Biosystems 3730.

RESULTS and DISCUSSION

Analysis of androgenic gland cDNA library

An unamplified library was established in plaque form. The titer unamplified λ lysate was 3 x 10⁶ pfu/ml and percentage of recombinant clones from blue/white colony calculation was 99%. The converted BM25.8 colonies were randomly picked to determine the insert by colony PCR using SP6 and T7 primers. The products of colony PCR were checked on 1.2% agarose gel as shown in Fig. 1.

![Figure 1](image1.png)

**Figure 1** PCR products from colony PCR using SP6 and T7 primers separated by electrophoresed on 1.2% agarose gel. Lane M is 100 bp ladder.

The 960 clones were subjected to single-pass DNA sequencing, resulting in 776 (78%) EST sequences. Identified clones generally shared a sequence identity >50% over a relatively long range (>150 bp) with the most similar sequence from BLASTX. Based on subjective criteria for gene identification at a minimum amino acid sequence similarity of expected value (E ≤ 10⁻⁴), 422 (65%) *M. rosenbergii* transcripts showed homology to previously described genes from *M. rosenbergii* and other species, including shrimp. ESTs matching with known genes were categorized into seven categories on the basis of general functions, storage protein (30.6%), gene/protein expression and modification (10.1%), hypothetical proteins (9.6%), cell/organism defense (8.3%), enzyme (2.1%), structural protein, cytoskeleton (0.5%), transport proteins (0.5%) and other (3.3%) as shown in Fig. 2.
A total of 266 transcripts did not share significant homology with any known sequence in the databases. Most of them are expected to be novel or specifically expressed in the androgenic gland. All sequences of no significant similarity were further analyzed by translating to protein sequences. The SMART domain search tool predicted the existence of a domain between residues 66-105 in one clone (which was subsequently named \textit{M. rosenbergii} androgenic gland-specific gene or \textit{Mr-AGs}, data bank accession number FJ539009) which was similar to insulin/insulin-like growth factor/relaxin family with low significant score (E-value = 2.17e+03). However, the Cq-IAG and three AGHs in isopods (Manor \textit{et al.}, 2007; Ohira \textit{et al.}, 2003; Okuno \textit{et al.}, 1997) are also the members of this family. Therefore, \textit{Mr-AGs} was proposed as a candidate gene for AGH.

Characterization of \textit{M. rosenbergii} androgenic gland-specific gene

The open reading frame of \textit{Mr-AGs} was 336 bp. The deduced amino acids and theoretical molecular weight of \textit{Mr-AGs} calculated by ProtParam program (ExPASy Proteomics Server) were 111 amino acids and 12.2 kDa, respectively. From alignment results from ClustalW, there was low similarity to the \textit{Armadillidium vulgare}, \textit{Porcellio scaber} and \textit{P. dillatatus} pro-AGH and pro-Cq-IAG (14, 10, 8 and 17\%, respectively) as shown in Fig. 3.

Since \textit{Mr-AGs} was similar to insulin/insulin-like growth factor/relaxin family which included AGH, the structure of \textit{Mr-AGs} was predicted by bioinformatic tools. The SignalP 3.0 result showed the existing of 16-amino acids signal peptide with max cleavage site probability of 0.507 while TargetP 1.1 result revealed that this signal peptide involved in the secretory pathway. The predicted signal...
peptide of Mr-AGs showed low similarity to the signal peptide of AGH from A. vulgar, P. scaber and P. dilatatus AGH and Cq-IAG signal peptides (25, 12, 12 and 6%, respectively, data not shown). No site of N-glycosylation was predicted in this sequence. There were 12 Cys residues in the sequence, but there were no prediction of disulfide bond forming. Although Mr-AGs is similar to insulin/insulin-like growth factor/relaxin family, its amino acids and molecular weight were quite different from P. scaber, P. dilatatus and A. vulgar AGH precursors as well as pro-Cq-IAG.

| Pos-AGH | --MEKLFLTVSLLCITLHQRWAVYQVNIGMESDVICADIFTVNHCIONELG--LFPPSRLLS 56 |
| Pod-AGH | --MEKLFLPSLVLFLTLHQRWAVYQVNIGMESDVICADIFTVNHCIONELG--RFPPARLT 56 |
| Arv-AGH | --MEKLFLTVSLLCITLHQRWAVYQVNIGMESDVICADIFTVNHCIONELG--YFPTLRLE 56 |
| Cq-IAG  | KLFLQLLNLILVVNLFPSPAFYVENVLLIDVBDCHLADTSICRTYQ--EFNTRAV 58 |
| F-D010  | --MENVVCLLALVNSAATKIVRPFPKPCAHCTNYLNSKXCGDGTQRPIFVE2HS 59 |

* * *

| Pos-AGH | KFCF-----------------------------UPNKRERSADDEYLFEDDEFPRALS-PP 99 |
| Pod-AGH | KFCF-----------------------------UPNKRERSADDEYLFEDDEFPRALSRT 94 |
| Arv-AGH | KFCF-----------------------------UPNKRERSADELYDEYDFPRALSIP 94 |
| Cq-IAG  | RCARDASFSVSVGDPGKGTVPRGAKLSKEKTYVFDARLGCEAATVDEAMT 110 |
| F-D010  | GICF-----------------------------ERPPFCF 70 |

* * *

| Pos-AGH | AAKSGERLEDIVSBSRSKRRDIAFHECCNIRTEHEKHKRTVECYC3RTYR----- 145 |
| Pod-AGH | AAKYDELEDIVSBSRSKRRDIAFHECCNIRTEHEKHKRTVECYC3RTYR----- 145 |
| Arv-AGH | EIEKMHKESDAFSLSGKRELAFHECCNIRTEHEKHKRTVECYC3RTYR----- 144 |
| Cq-IAG  | FIKTQYNMRRRSMTDNCCYTPYDCCSSEKATKCiYDFDQIEQDCELVSS 175 |
| F-D010  | ----RGTVAPALCAYVGCAMF3RCCWDCTLCYHTCPALF----- 111 |

**Figure 3** Multiple sequence alignment of F-D010 sequence with Armadillidium vulgar, Porcellio scaber and P. dilatatus pro-AGH and pro-Cq-IAG, by ClusatiW (Pos-AGH, Pod-AGH, Arv-AGH and Cq-IAG, respectively). The (-) indicates gaps introduced into the amino acid sequence to allow for the maximal degree of identity in the alignment.

**Tissue-specific analysis by qualitative RT-PCR**

The expression of the gene was studied in four tissues of five male prawns (AG, testis, vas deferens and hepatopancreas) and two tissues of five female prawns (ovary and hepatopancreas). The tissue-specific gene expression of Mr-AGs was examined by RT-PCR using specific primers. The expected size of the amplified cDNA fragment of Mr-AGs was 392 bp. High expression of Mr-AGs was detected in AGs from all male samples and low expression was detected in the vas deferens from two male samples. However, no signal was detected in testis, hepatopancreas in male and all female samples. An actin cDNA fragment as a positive control was detected in all tissues (Fig. 4).

In this research, RT-PCR analysis indicated that the expression of Mr-AGs was specific to the male prawn and mainly observed in AG and partially expressed in vas deferens. The localization of Mr-AGs mRNA indicated that this gene may play critical role in M. rosenbergii male. Standard histological techniques revealed that M. rosenbergii AG consists of epithelial cells attached to the
posterior vas deferens (Takuji and Hara, 2004). This tissue contaminant may cause the weak signals detected in the vas deferens. This result indicates that Mr-AGs expression might be androgenic gland-specific. Since the AGH mRNA in isopods from the previous studies was only expressed in AG (Ohira et al., 2003, Okuno et al., 1997), Mr-AGs is proposed to be the candidate of AGH. However, the biological activity assay of Mr-AGs protein or a gene-knockdown should be further investigated to confirm its identity.

(A) Mr-AGs

(B) Actin

Figure 4 In RT-PCR analysis, the first strand cDNAs were synthesized using total RNA from the androgenic gland (AG), testis (T), vas deferens (V), hepatopancreas (HP) from five male prawns, and ovary (O) and hepatopancreas (HP) from five female prawns. The expected size of the amplified cDNA fragments of Mr-AGs was 392 bp (A). As control, an actin cDNA fragments were amplified using specific primers in all tissues (B).

CONCLUSION

By screening a cDNA library of M. rosenbergii AGs, a gene which exhibited AG-specific expression pattern was identified. We proposed to call it Mr-AGs. The Mr-AGs is a novel gene since its sequence had no significant homology to any known genes or proteins. The localization of Mr-AGs
mRNA in the male AG indicates that this gene may play critical roles on sex regulation of male *M. rosenbergii*. This discovery provides important information for studying the AGH and AG-specific gene in *M. rosenbergii*.

ACKNOWLEDGEMENTS

This work was supported by grants from Kasetsart University Research and Development Institute (KURDI) 2005 and 2007, TRF-Master research grants (2006), and Research Grants for Master Degree student from the Graduate School, Kasetsart University (2006).

REFERENCES


