

## CONTENTS

	<b>Page</b>
<b>ACKNOWLEDGEMENTS</b>	<b>iii</b>
<b>ABSTRACT (ENGLISH)</b>	<b>iv</b>
<b>ABSTRACT (THAI)</b>	<b>v</b>
<b>LIST OF TABLES</b>	<b>xi</b>
<b>LIST OF FIGURES</b>	<b>xii</b>
<b>LIST OF ABBREVIATIONS</b>	<b>xv</b>
<b>CHAPTER I INTRODUCTION</b>	
1.1 Reproductive biology of <i>Penaeus monodon</i>	1
1.2 Neurohormonal regulation of female crustacean's reproduction	2
1.3 The role of dopamine neurotransmitter	3
1.4 Characteristic of G-protein coupled dopamine receptor	4
1.5 Signaling pathway of GPCRs	5
1.6 Classification of DARs	8
1.6.1 DARs in vertebrates	8
1.6.2 DARs in invertebrate	9
1.6.3 Functional properties and signaling transduction of DARs	9
1.7 Distribution and localization of DARs	12
<b>CHAPTER II OBJECTIVES</b>	<b>13</b>
<b>CHAPTER III MATERIALS</b>	
3.1 Shrimp samples	14
3.2 Bacterial strains	14
3.3 Mammalian cell	14
3.4 Culture medium	14
3.4.1 Bacterial cell culture medium	14
3.4.2 COS-1 cell culture medium	15
3.5 Plasmid vectors	15

## CONTENTS (cont.)

	<b>Page</b>
3.6 Modification and restriction enzymes	15
3.7 Oligonucleotide primers	16
3.8 Miscellaneous	18
 <b>CHAPTER IV METHODS</b>	
4.1 Total RNA extraction	22
4.2 RNA electrophoresis	22
4.3 Rapid Amplification of cDNA ends (RACE)	23
4.3.1 Amplification of 3' end of cDNA by 3'RACE	23
4.3.2 Amplification of 5' end of cDNA by 5'RACE	25
4.3.3 Amplification of the coding sequence of <i>P. monodon</i> 's dopamine receptor (DAR_Pem) cDNA	25
4.4 Cloning of cDNA fragments	27
4.4.1 Purification of PCR product fragments and plasmid DNA by QIAquick Gel Extraction Kit (QIAGEN)	27
4.4.2 Agarose gel electrophoresis	27
4.4.3 DNA Ligation	28
4.4.4 Competent <i>Escherichia coli</i> cells preparation	28
4.4.5 Recombinant plasmid DNA Transformation by heat-shock	29
4.5 Verification of recombinant plasmid DNA	29
4.5.1 Recombinant plasmid DNA determination by colony PCR	29
4.5.2 Recombinant plasmid DNA determination by size screening	29
4.5.3 Recombinant plasmid DNA determination by restriction enzyme digestion	30
4.5.3.1 Plasmid DNA extraction by CTAB mini-preparation	30
4.5.3.2 Restriction enzyme digestion of plasmid DNA	30
4.5.4 Determination of recombinant plasmid by sequencing	31
4.5.5 Analysis of DAR structure by computer	31

## CONTENTS (cont.)

	<b>Page</b>
4.6 Determination of DAR_Pem mRNA expression level in the black tiger shrimp	31
4.6.1 Shrimp sample preparation	31
4.6.2 Determination of DAR_Pem mRNA expression by RT-PCR	31
4.6.3 DAR_Pem mRNA expression analysis	32
4.7 Expression of recombinant C-terminal tail of <i>P. monodon</i> 's dopamine receptor (C-tail DAR)	32
4.7.1 Construction of recombinant plasmid for expression of C-tail DAR	32
4.7.2 Protein expression of C-tail DAR by <i>Escherichia coli</i> expression system	33
4.7.3 Protein expression analysis by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)	33
4.7.3.1 Separation of protein fractions	33
4.7.3.2 Soluble fraction preparation	34
4.7.3.3 Preparation of protein sample for gel loading	34
4.7.3.4 SDS-PAGE preparation	34
4.8 Polyclonal antibody production	35
4.8.1 Protein purification by Electro-Eluter (Model 422, BIO-RAD)	35
4.8.2 Polyclonal antibody production in rabbit	36
4.9 Sensitivity and specificity of polyclonal antibody	36
4.9.1 Dot blot analysis	36
4.9.2 Western blot analysis	36
4.10 Determination of DAR expression protein in black tiger shrimp	37
4.10.1 Total protein extraction	37
4.10.2 Membrane protein extraction	37
4.10.3 Measurement of protein concentration by Bradford method	38

## CONTENTS (cont.)

	<b>Page</b>
4.11 Expression of DAR_Pem in COS-1 cell	38
4.11.1 Construction of DAR_Pem expression plasmid	38
4.11.2 COS-cell culture	38
4.11.3 Plasmid DNA purification by plasmid DNA extraction with mini prep QIAquick Kit (Qiagen)	39
4.11.4 Plasmid DNA transfection	39
4.12 Immunofluorescent	39
4.13 cAMP assay	40
4.13.1 Activation of biogenic amine to DAR_Pem with biogenic amine	40
4.13.2 Measurement of cAMP level	40
 <b>CHAPTER V RESULTS</b>	
5.1 Total RNA extraction	42
5.2 Rapid Amplification of cDNA ends (RACE)	42
5.2.1 Amplification of the 3' end cDNA by 3'RACE	42
5.2.2 Analysis of 5' end cDNA amplification by 5'RACE	47
5.3 Assembly of DAR_Pem cDNA sequence	47
5.4 Amplification of coding DAR_Pem cDNA sequence	53
5.5 analysis of the Pem_DAR cDNA sequence	53
5.6 Determination of DAR mRNA expression levels in <i>P. monodon</i>	54
5.6.1 Determination of female <i>P. monodon</i> 's ovarian stages	54
5.6.2 Determination of DAR mRNA expression levels	62
5.7 Protein expression and polyclonal antibody production	65
5.7.1 Cloning of C-tail DAR fragment into pET-17b expression vector	65
5.7.2 Expression of recombinant C-tail DAR in <i>E. coli</i> expression system	70
5.7.3 Polyclonal antibody production	70
5.8 Determination of specificity and sensitivity of C-tail DAR antibody (anti C-tail DAR) by dot blot and western blot analysis	74

**CONTENTS (cont.)**

	<b>Page</b>
5.9 Protein expression analysis of DAR_Pem black tiger shrimp	74
5.9.1 Analysis of DAR_Pem expression in total proteins from shrimp tissues	74
5.9.2 Analysis of DAR_Pem expression in the membrane protein fraction of shrimp tissues	77
5.9.3 Determination of cross-reactivity analysis of pre-immunized serum	77
5.10 Construction of DAR_Pem expression cassette in pcDNA 3.1 (+) vector	77
5.11 Expression of DAR_Pem in COS-1 cell1	82
5.11.1 Detection of DAR_Pem transcript in COS-1 cells	82
5.12.2 Detection of DAR_Pem protein in COS-1 cells	87
5.12 Determination of DAR_Pem protein expression and localization by immunofluorescence microscopy	87
5.13 Determination of activity of DAR_Pem by cAMP assay	87
<b>CHAPTER VI DISCUSSION</b>	<b>93</b>
<b>CHAPTER VII CONCLUSION</b>	<b>99</b>
<b>REFERENCES</b>	<b>100</b>
<b>BIOGRAPHY</b>	<b>107</b>

## LIST OF TABLES

<b>Table</b>	<b>Page</b>
1.1 Classification and general function of DARs	11
3.1 Description of restriction enzymes (Promega) for recombinant plasmid DNA digestion	16
3.2 List of nucleotide sequences and T <sub>m</sub> of primers	17
4.1 SDS-PAGE components	35
5.1 The percentage of GSI values and ovarian developmental stages of female <i>P. monodon</i>	62

## LIST OF FIGURES

<b>Figure</b>	<b>Page</b>
1.1 Biosynthesis of dopamine (DA)	4
1.2 The signaling pathway of G-protein coupled receptor (GPCRs)	7
3.1 Physical map of pGEM <sup>®</sup> -T Easy vector (Promega)	19
3.2 Physical map of pET-17b vector map (Novagen)	20
3.3 Physical map of pcDNA 3.1 (+) vector and multiple cloning sites (Modified from Invitrogen)	21
4.1 Amplification of the 3' end of putative DAR cDNA	24
4.2 A schematic diagram of 5' end amplification of putative DAR cDNA by 5' RACE	26
5.1 Total RNA from wild female broodstock <i>P. monodon</i> at ovarian developmental stage I	43
5.2 The 3'RACE PCR product of the putative DAR_Pem cDNA	44
5.3 Purification of 3'RACE fragments of the putative DAR_Pem cDNA	45
5.4 Verification of recombinant clones containing 3' cDNA end fragment of the putative DAR_Pem by <i>EcoR</i> I digestion	46
5.5 Nucleotide sequence alignment of 3'RACE clones of the putative DAR_Pem cDNA	48
5.6 The 5'RACE PCR product of the putative DAR_Pem cDNA	49
5.7 Verification of recombinant clones containing 5' cDNA end fragment of the putative DAR_Pem cDNA by <i>EcoR</i> I enzyme digestion	50
5.8 Nucleotide sequence alignment of 5' RACE fragments of the putative DAR_Pem	51
5.9 The combined nucleotide sequence between the 3' RACE and 5' RACE of the putative DAR_Pem cDNA	52
5.10 Amplification and purification of a cDNA fragment encoding DAR_Pem	55

## LIST OF FIGURES (cont.)

<b>Figure</b>	<b>Page</b>
5.11 Verification of recombinant plasmid harboring a coding DAR_Pem cDNA by <i>EcoR</i> I digestion	56
5.12 Nucleotide sequence alignment of cDNA encoding DAR_Pem	57
5.13 Amino acid sequence alignment of cDNA encoding DAR_Pem	58
5.14 Hydrophobic plot of the deduced amino acid sequence of DAR_Pem	59
5.15 Nucleotide and deduced amino acid sequence of the full-length DAR_Pem	60
5.16 Alignment of amino acid sequences of type 1 DARs	61
5.17 The expression levels of DAR mRNA in various tissues of wild <i>P. monodon</i> 's broodstock at ovarian developmental stage I-IV	63
5.18 Comparison of DAR mRNA expression in various tissues of wild <i>P. monodon</i> 's broodstock at ovarian developmental stage I-IV	64
5.19 PCR amplification of the C-tail DAR fragment	66
5.20 Screening of recombinant clones harboring C-tail DAR fragment in pET-17b by colony PCR	67
5.21 Determination of recombinant clones harboring C-tail DAR fragment In pET-17b with <i>Nde</i> I and <i>EcoR</i> I digestion	68
5.22 Nucleotide sequence alignment of the C-tail DAR fragment	69
5.23 Expression of the recombinant C-tail DAR by <i>E. coli</i> expression system	71
5.24 Analysis of the recombinant C-tail DAR protein in different fraction of <i>E. coli</i> cells	72
5.25 Purification of the recombinant C-tail DAR protein	73
5.26 Sensitivity and specificity of anti C-tail DAR antibody	75
5.27 Detection of DAR expression in total proteins of shrimp tissues	76
5.28 Detection of DAR expression in the membrane fraction of female shrimp tissues by western blot	78

**LIST OF FIGURES (cont.)**

<b>Figure</b>	<b>Page</b>
5.29 Cross-reactivity of rabbit pre-immunized serum to shrimp ovarian membrane proteins	79
5.30 Cross-reactivity between pre-immunized serum from rabbit and mouse with extracted protein from shrimp tissues	80
5.31 Amplification of the coding DAR_Pem cDNA fragment by <i>Pfu</i> DNA polymerase	81
5.32 Purification of pcDNA 3.1 (+) vector and coding DAR_Pem cDNA fragment	83
5.33 Determination of recombinant clones harboring the coding DAR_Pem cDNA by rapid size screening	84
5.34 Determination of recombinant clones harboring the DAR_Pem cDNA by <i>EcoR</i> I and <i>Hind</i> III digestion	85
5.35 Detection of DAR_Pem mRNA expression in COS-1 cell	86
5.36 Determination of DAR_Pem expression in COS-1 cells	89
5.37 Immunofluorescence detection of DAR_Pem protein expression by anti-DAR primary antibody and FITC conjugated secondary antibody	90
5.38 Cyclic AMP assay (experiment 1)	91
5.39 Cyclic AMP assay (experiment 2)	92

## LIST OF ABBREVIATIONS

APS	Ammonium persulphate
°C	degree Celsius
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
CTAB	cetyl trimethyl ammonium bromide
C-terminal	carboxy terminal
DA	Dopamine
DAR	Dopamine receptor
DAR_Pem	<i>Penaeus monodon</i> 's dopamine receptor
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
<i>E. coli</i>	<i>Escherichia coli</i>
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GIH	Gonad-stimulating hormone
GPCR	G-protein couple receptor
GSH	Gonad-stimulating hormone
GSI	gonado-somatic index
hr	hour
IPTG	isopropyl- $\beta$ -D-thiogalactopyranoside
kb	kilobase pair (s)
kDa	kilodalton (s)
LB	Luria-Beriani (medium)
<i>M. rosenbergii</i>	<i>Macrobrachium rosenbergii</i>
min	minute (s)

**LIST OF ABBREVIATION (cont.)**

ng	nanogram
nm	nanometer (s)
OD <sub>600</sub>	optical density at 600 nm
ORF	open reading frame
<i>P. monodon</i>	<i>Penaeus monodon</i>
PBS	phosphate buffer saline
PCR	polymerase chain reaction
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
RACE	Rapid Amplification of cDNA ends
RNase	ribonuclease
rpm	revolutions per minute
RT	reverse transcriptase
sec	second
S.E.M.	standard deviation
TEMED	N,N,N,N'- tetramethyl-ethylenediamide
UTR	untranslated regions
U	unit (s)
UV	ultraviolet
v/v	volume / volume
w/v	weight / volume