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Molecular characterization of a cDNA encoding red pigment-concentrating hormone in black tiger shrimp *Penaeus monodon*: Implication of its function in molt and osmoregulation

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ABSTRACT

Red pigment-concentrating hormone (RPCH) is a member of the AKH/RPCH peptide family present mainly in crustaceans and insects. Insect AKH is responsible for metabolic functions whereas RPCH plays a major role in the aggregation of red chromatophores in crustaceans. In this study, a full-length cDNA of RPCH of the black tiger shrimp, *Penaeus monodon* (*PmRPCH*) was cloned by Rapid Amplification of cDNA Ends strategies from the eyestalk RNA. A 770 bp full-length *PmRPCH* cDNA harbored 279 bp of an open reading frame encoding a signal peptide of 21 amino acid residues, an 8 amino acid mature RPCH peptide, followed by 61 amino acid residues of a RPCH precursor-related peptide. The highest levels of *PmRPCH* mRNA expression were detected in eyestalks while lower expression was found in other nervous tissues i.e. brain, thoracic ganglia and abdominal nerve cord. Expression of *PmRPCH* was transiently stimulated upon hypersalinity change within 12 h suggesting its osmoregulatory function. During the molting cycle, *PmRPCH* in the eyestalk was expressed at the lowest level in the early pre-molt stage (D_0), then gradually increased over the pre-molt period and reached the highest level in the late pre-molt (D_4) and post-molt (AB) stages. RPCH peptide at a dose of 100 pmol also increased gill Na^+/K^+ ATPase activity in 36–48 h after injection. However, PmRPCH did not accelerate the duration of molting cycle. Our results provide the first evidence on the potential function of PmRPCH in molting, probably by mediating hemolymph osmolality and ion transport enzymes during the late pre-molt stage.

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1. Introduction

Red pigment-concentrating hormone (RPCH) is an octapeptide that belongs to the adipokinetic hormone (AKH)/red pigment-concentrating hormone (RPCH) family. The primary structure of a mature RPCH and AKH is typically composed of 8–10 amino acid residues with an N-terminal pyroglutamate and a C-terminal amidation (Gäde, 2009). In crustaceans, RPCH was mostly presented in diverse members of the order Decapoda. The crustacean RPCH was majorly expressed in nervous tissues such as the X-organ (XO) in the eyestalk (Mangerich et al., 1986; Alvarado-Alvarez et al., 1999; Chung and Webster, 2004), brain, and thoracic ganglia (Mangerich et al., 1986; Nusbaum and Marder, 1988; Chung and Webster, 2004).

The RPCH primarily functions in mediating pigment aggregation in crustaceans (Rao, 2001). Alvarado-Alvarez et al., 1999 demonstrated the biological activity of a synthetic RPCH to aggregate the tegumentary erythrophores of a crayfish *Procambarus clarkii* in vitro (Alvarado-Alvarez et al., 1999). Thereafter, several studies were successful to demonstrate in vivo the chromatophorotropic activity of the synthetic RPCH on pigment aggregation (Ribeiro and McNamara, 2009; Marco and Gäde, 2010). RPCH triggered pigment aggregation via the Ca^{2+}-activated cGMP signaling cascade. In the red ovarian chromatophores of the fresh water shrimp *Macrobrachium olfersi*, both the release of Ca^{2+} from smooth endoplasmic reticulum (SER) and the blockage of K^+ influx led to membrane depolarization and thus allowed phosphorylation of downstream molecular motors essential for pigment aggregation (Milograna et al., 2010).

In addition to its major role in pigment aggregation, RPCH has also been reported to participate in other physiological processes. For instance, RPCH of the crayfish *P. clarkii* was shown to mediate retinal responsiveness to the circadian rhythm (Smith and Naylor, 1972). Sarojini et al. (1995) demonstrated that RPCH was involved in the reproductive system as a neurotransmitter that triggered the release of gonad-stimulating hormone (GSH) in the brain and/or thoracic ganglia of *P. clarkii*. In addition, a metabolic function of RPCH was investigated in an isopod, *Porcellio scaber*, where it triggered an increase in glucose level in the hemolymph (Zralá et al., 2010). These studies indicated that RPCH plays multifunctional roles in crustaceans. In *Penaeus*

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monodon, an amino acid sequence of the mature RPCH peptide (GenBank accession no. B3EWG0) is available but its cDNA has not yet been characterized.

Molt or ecdysis is an essential process for crustacean growth. The molting process involves several physiological mechanisms such as hormonal control, osmoregulation and metabolism (Chang and Mykles, 2011). Lipid and glucose were stored as an energy source during the intermolt stage, and were highly metabolized to supply energy for several metabolic processes during the pre-molt to post-molt stages (Siegert et al., 1993; Chamberlin et al., 1997; Galindo et al., 2009). Up-regulation of ion transport was found when the animals were just about to molt. Subsequently, water uptake became a major event during the period of post-molt (Jasmani et al., 2008; Galindo et al., 2009; Wilder et al., 2009; Jasmani et al., 2010; Bonilla-Gómez et al., 2012).

In this study a cDNA encoding RPCH of the black tiger shrimp *P. monodon* was cloned and characterized. According to its diverse function in other organisms, *PmRPCH* was investigated for its involvement in related physiological processes including salinity change and osmoregulation. In addition, our study provides the first evidence for a possible function of RPCH in the molting process of crustaceans.

2. Materials and methods

2.1. RNA extraction and cDNA synthesis

Black tiger shrimp *P. monodon* were kindly provided by Shrimp Genetic Improvement Center, Surat Thani, Thailand. They were acclimated in 5 ppt seawater until used. Shrimp tissues were freshly dissected and used for RNA extraction by Ribozol (Ameresco, USA) following the manufacturer's protocol. The amount of total RNA was determined from the absorbance at 260 nm as measured by Nanodrop 1000 (Thermo-scientific, USA). To synthesize a first-strand cDNA, 1 μg of the total RNA was mixed with 50 nM of oligo-dT primer and heated at 70 °C for 5 min then, snap cooled on ice for 3 min. The mixture composing of 1X RTase buffer, 3 mM $MgCl_2$, 0.4 mM dNTP and 1 μl of reverse-transcriptase ImpromII (Promega, USA) was added into the preheated RNA. The reaction was then incubated at 25 °C for 5 min, 42 °C for 60 min and 70 °C for 10 min. The cDNA was kept at −20 °C.

2.2. Cloning of a full-length PmRPCH cDNA

A full-length cDNA of *PmRPCH* was obtained by the Rapid Amplification of cDNA Ends (RACE) strategy using the first-strand cDNA from shrimp eyestalk. In 3′RACE, 1 μl of the oligo-dT-primed first-strand cDNA was amplified with 0.2 μM of 3RACE-PmRPCH-F1 and oligo-dT primers in a 25 μl reaction containing 1X *Taq* buffer + $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.2 mM dNTP and 1 U *Taq* polymerase (Thermo-scientific, USA). Specific product was subsequently obtained by nested amplification using the first PCR product as a template with 3RACE-PmRPCH-F2 and PM1 primers. In 5′RACE, the first-strand cDNA was synthesized by 5RACE-PmRPCH-R1 and then tailed with dATP at the 3′end by terminal deoxytransferase (Promega, USA) using the manufacturer's protocol. The A-tailed cDNA was amplified with 5RACE-PmRPCH-R2 and oligo-dT primers in a reaction containing the same mixture as the 3′RACE reaction. Then, nested PCR was performed with the same condition as the first PCR but with 5RACE-PmRPCH-R3 and PM1 primers. The expected PCR products were ligated to a pGEM-T easy vector (Promega, USA) then, transformed into *Escherichia coli* DH5α. The nucleotide sequences of recombinant clones were determined by automated DNA sequencing at First-base Co., Ltd (Malaysia). The coding sequence of a full-length *PmRPCH* was verified by PCR using 3RACE-PmRPCH-F2 and 3UTR-PmRPCH-R primers with Phusion® high-fidelity DNA polymerase (Finnzymes, USA). Nucleotide sequences of all primers in the cloning steps are listed in Table 1.

Table 1
Primer pairs used in the experiments.

Name	Objectives	Sequence (5′–3′)
3RACE-PmRPCH-F1	3′RACE	CGGAACCATCCCAGTCACAAC
3RACE-PmRPCH-F2	3′RACE	CTG GGCAAGCGAGCAGCCG
5RACE-PmRPCH-R1	5′RACE	TGATAAGYCKGTAGATGTGCATGAC
PmRPCH-R2	5′RACE and RT-PCR	TGACGGTTGTGACTG GGATGG
PmRPCH-R3	5′RACE	TGCGGCTGCTCGCTTGCC C
PmRPCH-F	RT-PCR	CAGATATGGTTCGTGCCGTCG
3UTR-PmRPCH-R	RT-PCR	ACGAAACGGTAGATGGTTGTTG
Ef-1α-F	RT-PCR	GAACTGCTGACCAAGATCGACAGG
Ef-1α-R	RT-PCR	GAGCATACTGTTGGAAGGTCTCCA
ActinF	RT-PCR	GACTCGTACGTGGGCGACGAGG
ActinR	RT-PCR	AGCAGCGGTGGTCATCTCCTGCTC

2.3. Determination of PmRPCH expression in shrimp tissues

Adult female *P. monodon* (approximately 100 g) were anesthetized on ice and various tissues including the eyestalk, brain, thoracic ganglia, abdominal nerve cord, hepatopancreas, gill, lymphoid, muscle and hemocytes were freshly collected for RNA isolation. One microgram of the total RNA was used for cDNA synthesis, and semi-quantitative RT-PCR was performed to determine the expression of *PmRPCH* with PmRPCH-F and PmRPCH-R2 (Table 1) primers using the following temperature cycles: 94 °C for 3 min, then 26 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. A beta-actin transcript was amplified by Actin-F and Actin-R primers as an internal control.

2.4. Reverse transcription real-time PCR

The *PmRPCH* mRNA levels in shrimp eyestalk in subsequent experiments were determined by reverse transcription relative real-time PCR. The appropriate dilution of oligo-dT-primed cDNA was amplified with PmRPCH-F and PmRPCH-R2 primers. The PCR mixture was composed of a 1X KAPA SYBR Fast ABI Prism qPCR kit (KAPA Biosystems, USA), and 0.25 μM of each primer. The reaction was subjected to amplification in a real-time PCR machine (realplex[4], Eppendorf) using the PCR profile of 95 °C for 3 min, then 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Elongation factor-1 alpha (*EF-1α*) was amplified as an internal control with EF1α-F and EF1 α-R primers using the same PCR profile. Each sample was amplified in duplication. The purified PCR products of both *PmRPCH* and *EF-1α* were diluted to 10^2–10^8 copies and used as a template to establish a standard curve for each gene. The threshold cycle of both genes was calculated as copy number, and then the copy number of *PmRPCH* was normalized with that of *EF-1α*.

2.5. Expression of PmRPCH upon hypersalinity change

Shrimp (approximately 5 g) in the early pre-molt stage (D_0) were acclimated in 5 ppt seawater at 28–30 °C for one week before use. Two independent experiments were performed. The shrimp were divided into two groups; each group contained 5 shrimp. At the beginning of the experiment shrimp in the control group and the experimental group were transferred from 5 ppt seawater to a new tank filled with 5 ppt and 30 ppt seawater, respectively. Shrimp eyestalks from each group were collected at 6 h, 12 h, 24 h, 36 h and 48 h (n = 5 for each time point) and determined for *PmRPCH* expression by reverse transcription real-time PCR.

2.6. Expression of PmRPCH and gill-Na^+–K^+ ATPase activity during the molting cycle

Shrimp (approximately 10 g) were reared in 5 ppt seawater at 28–30 °C. The molting stages of the shrimp were divided into intermolt (C), early pre-molt (D_0), pre-molt (D_{2-3}), late pre-molt (D_4) and

post-molt (AB) stages (Promwikorn et al., 2004). *PmRPCH* mRNA expression levels were determined by reverse transcription real-time PCR. Two independent experiments were performed; each experiment was composed of three shrimp at each molting stage. Anterior (1st to 4th) and posterior (5th to 8th) gills were also collected for Na^+–K^+ ATPase activity assay.

2.7. Measurement of Na^+/K^+ ATPase activity in the gill

Activity of Na^+/K^+ ATPase in both anterior and posterior gills was measured by following Holliday's method (Holliday, 1985). In brief, freshly isolated gills were washed twice in cold-SEI buffer (250 mM sucrose, 10 mM EDTA and 50 mM imidazole pH 7.4) then, homogenized in 10-volumes of cold-SEI buffer containing 0.1% sodium deoxycholate. The cell debris was eliminated by centrifugation at 600 *g*, 4 °C for 5 min. The supernatant was further centrifuged at 10,000 *g* at 4 °C for another 10 min. Protein concentration in the supernatant was measured by Bradford assay (Invitrogen) before Na^+/K^+ ATPase activity assay. Ten microliters of each sample was added to the salt solution (130 mM NaCl, 30 mM KCl and 50 mM imidazole pH 7.4) or salt solution containing ouabain, a Na^+/K^+ ATPase inhibitor (130 mM NaCl, 1 mM ouabain and 50 mM imidazole pH 7.4). The reaction was pre-incubated at 37 °C for 5 min, then 0.7 mM ATP/7 mM $MgCl_2$ was added and incubated for 20 min. The reaction was stopped by adding 1 mL of cold-stop solution (8.4 mM ammonium molybdate, 3.2% v/v H_2SO_4 and 4.8% w/v $FeSO_4$). The concentration of phosphate was measured at OD_{700}. The activity unit was calculated in μmol Pi/mg protein/h.

2.8. Effect of synthetic RPCH on gill Na^+/K^+ ATPase activity

A synthetic RPCH (pyroGlu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-NH_2) at approximately 90% of purity was produced from ChinaPeptides Company, China. The synthetic peptide was dissolved in 80% methanol to a final concentration of 1 mg/mL. The working synthetic RPCH was freshly diluted with 150 mM NaCl before use. Shrimp in the early pre-molt (D_0) stage reared in 5 ppt seawater were injected with 10 pmol and 100 pmol RPCH peptide, or 150 mM NaCl as a control group ($n = 5$ for each group). Anterior and posterior gills were isolated to measure Na^+/K^+ ATPase activity at different time points after injection (6–48 h).

2.9. Effect of synthetic RPCH on molt duration in shrimp

Shrimp (approximately 10 g) reared in 5 ppt seawater at 28–30 °C were allowed to molt. Three days after molt, shrimp at the late intermolt stage ($n = 7$) were injected with a single dose of synthetic RPCH at the doses of 1 pmol, 10 pmol and 100 pmol or with 150 mM NaCl. In addition, shrimp were also injected with 100 pmol synthetic RPCH or NaCl daily for 6 days. The molt duration of shrimp in both groups was recorded.

2.10. Statistical analysis

The expression of eyestalk *PmRPCH* under hypersalinity changes and gill Na^+/K^+ ATPase activity responding to RPCH peptide were determined for significant difference by two-way ANOVA and Bonferroni's test. The expression level of eyestalk *PmRPCH* mRNA and gill Na^+/K^+ ATPase activity in different molt stages were analyzed by one-way ANOVA and compared between groups by Duncan's test. Data analysis was performed using SPSS Statistic version 20 (IBM®, USA).

3. Results

3.1. Cloning and characterization of PmRPCH cDNA

PCR fragments of 480 bp and 358 bp were obtained from 3′ and 5′ RACE, respectively with an overlapping region of 68 bp. The combined sequence of *PmRPCH* cDNA was composed of a 129 bp 5′UTR, a 279 bp of coding region that encoded for 93 amino acid residues and a 3′UTR of 362 bp. The entire coding sequence of PmRPCH (GenBank Accession No. KC757347) was subsequently verified by PCR and characterized. The deduced amino acid sequence of PmRPCH possessed a signal peptide in the first 21 amino acid residues as predicted by VSA-QL (SignalP 4.0). The mature peptide of PmRPCH was composed of eight amino acid residues with the sequence Gln-Leu-Asn-Phe-Ser-Pro-Gly-Trp. A potential amidation site was found at the C-terminus of the mature peptide followed by a dibasic cleavage site (GKR) that, after cleavage, would produce an RPCH precursor-related peptide of 61 amino acid residues (Fig. 1). Amino acid sequence alignment showed that the PmRPCH mature peptide sequence was identical to that of RPCH of other crustaceans, whereas some variations were presented in the precursor-related peptide region. In addition, an alignment of the mature PmRPCH to the mature insect AKH peptides indicated highly conserved amino acid residues; Gln^1, Leu^2, Phe^4 and Trp^8 (Fig. 1).

3.2. Tissue distribution of PmRPCH expression

The *PmRPCH* mRNA expression levels in various shrimp tissues were determined by semi-quantitative RT-PCR. The result in Fig. 2 showed that *PmRPCH* mRNA was highly expressed in the eyestalk. Lower levels of *PmRPCH* expression could be detected in the brain, thoracic ganglia and abdominal nerve cord whereas the expression in other tissues i.e. hepatopancreas, lymphoid, gill, muscle and hemocytes was hardly detectable.

3.3. Expression of PmRPCH in response to hypersalinity change

To determine the effect of stress condition caused by hypersalinity change on *PmRPCH* expression, shrimp that had been acclimated in low salt seawater (5 ppt) were exposed to high salt seawater (30 ppt) for 6, 12, 24, 36 and 48 h. The *PmRPCH* mRNA expression level was determined by quantitative real-time PCR. The result in Fig. 3 showed that an almost three-fold change of the *PmRPCH* mRNA level was observed in the shrimp that were exposed to 30 ppt seawater for 12 h compared with that in the control shrimp that were continuingly reared in 5 ppt seawater ($p < 0.05$), before gradually declining at 24–48 h. In contrast, the expression of *PmRPCH* in the control shrimp reared in 5 ppt seawater was not changed over time courses ($p > 0.05$).

3.4. Relationship between PmRPCH expression and gill Na^+/K^+ ATPase activity during the molting cycle

The expression profile of *PmRPCH* mRNA in shrimp eyestalks at each molting stage was determined to investigate the involvement of PmRPCH in the molting process. The result showed that the *PmRPCH* mRNA level was lowest at the early pre-molt stage (D_0), then gradually increased and reached the highest level at the late pre-molt stage (D_4) and post-molt stage (AB); an approximately 4-fold increase ($p < 0.05$) compared to that in the D_0 stage. The expression of *PmRPCH* was then decreased after molt and remained at a low level throughout the intermolt (C) stage (Fig. 4A). In addition, gill Na^+/K^+ ATPase activity during the molting cycle was determined. The Na^+/K^+ ATPase activity in both anterior and posterior gills (Fig. 4B and C, respectively) showed the lowest activity (45.5 ± 5.8 and 35.0 ± 4.2 μmol Pi/mg protein/h, respectively) at the pre-molt (D_{23}) stage. The activity was significantly raised in the late pre-molt stage (D_4; 118.6 ± 25.7 μmol Pi/mg protein/h) and post-molt stage (AB; 98.1 ± 9.1 μmol Pi/mg protein/h) in posterior gills, whereas the Na^+/K^+ ATPase activity in anterior gills remained at low levels over pre-molt stages before increasing in the post-molt stage (124.4 ± 25.4 μmol Pi/mg protein/h).

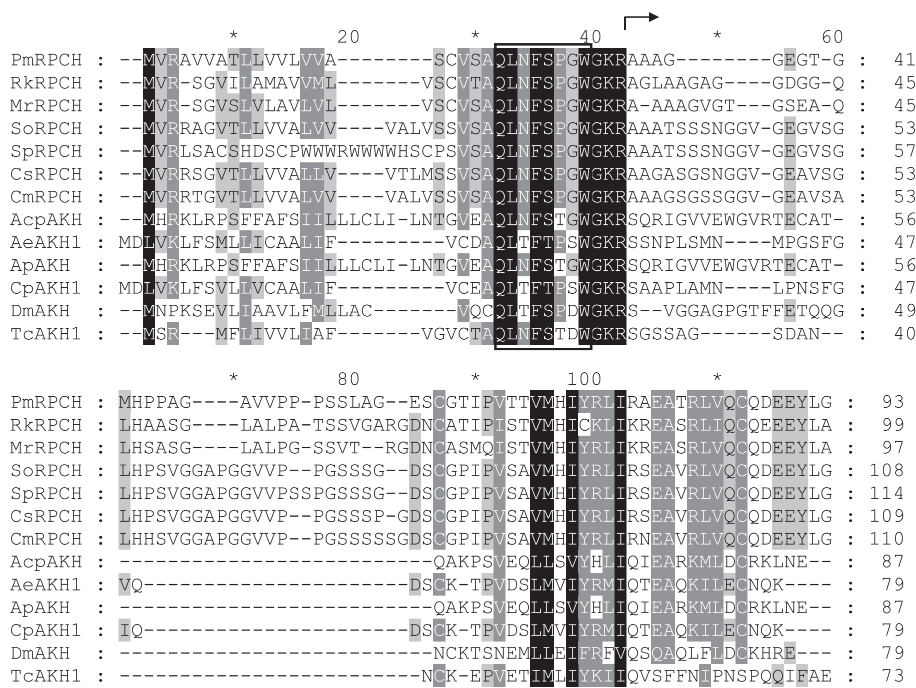

Fig. 1. Amino acid sequence alignment of PmRPCH with RPCH/AKH from other species. The deduced amino acid sequence of PmRPCH (GenBank accession no. KC757347) is aligned with RPCH from *Rimicaris kairei* (RkRPCH; ACZ51370), *Macrobrachium rosenbergii* (MrRPCH; ABV46765), *Scylla olivacea* (SoRPCH; ADQ73633), *Scylla paramamosain* (SpRPCH; AGW45011), *Callinectes sapidus* (CsRPCH; AAC37244) and *Carcinus maenas* (CmRPCH; AAB28133), and with insect-AKH from *Acyrthosiphon pisum* (AcpAKH; AEY77127), *Aedes aegypti* (AeAKH1; CAY77165), *Apis mellifera* (ApAKH; AEW68342), *Culex pipiens* (CpAKH1; CAY77163), *Drosophila melanogaster* (DmAKH; NP_523918), and *Tribolium castaneum* (TcAKH1; ABN79648). Amino acids that are identical or conserved in all sequences are highlighted in black color. Box and arrow indicate sequences of mature RPCH/AKH peptides and precursor-related peptide, respectively.

3.5. Effect of synthetic RPCH on gill Na^+/K^+ ATPase activity

The effect of synthetic RPCH on Na^+/K^+ ATPase activity in both anterior and posterior gills were determined by Na^+/K^+ ATPase activity assay. The result in Fig. 5 showed that the Na^+/K^+ ATPase activity in both anterior (A) and posterior (B) gills did not change after RPCH injection, comparing with NaCl injection in the first 24 h. At 36 h after injection, the anterior gill Na^+/K^+ ATPase activity was significantly increased in the shrimp injected with 100 pmol RPCH (118.9 ± 16.3 μmol Pi/mg protein/h) compared with that in NaCl-injected shrimp (49.7 ± 5.4 μmol Pi/mg protein/h). Similarly, the Na^+/K^+ ATPase activity in posterior gills of 100 pmol RPCH-injected shrimp (84.6 ± 10.3 μmol Pi/mg protein/h) was significantly higher than that in NaCl injected shrimp (63.4 ± 6.2 μmol Pi/mg protein/h). The posterior gill Na^+/K^+ ATPase activity in 100 pmol-RPCH injected shrimp continued with a significant increase at 48 h after injection (91.4 ± 12.6 μmol Pi/mg protein/h) compared with the control shrimp (47.7 ± 4.6 μmol Pi/mg protein/h).

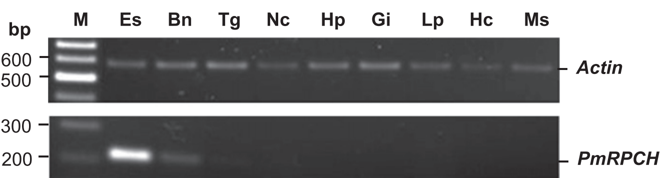

Fig. 2. Expression of *PmRPCH* mRNA in shrimp tissues. The expression levels of *PmRPCH* were detected by semi-quantitative RT-PCR. A representative of RT-PCR product of PmRPCH in eyestalk, Es; brain, Bn; thoracic ganglia, Tg; abdominal nerve cord, Nc; hepatopancreas, Hp; gill, Gi; lymphoid, Lp; hemocyte, Hc and muscle, Ms from one adult female *P. monodon* is shown. The upper bands (approximately 550 bp) represent the *β-actin* transcript that was used as an internal control and the lower bands (approximately 210 bp) represent *PmRPCH* RT-PCR products in each tissue. M is a 100 bp DNA ladder.

3.6. Effect of synthetic RPCH on molt duration

To determine whether the molting cycle was mediated by RPCH peptide, shrimp were injected with the synthetic RPCH and the duration of molt was recorded and compared to that of the shrimp injected with NaCl. Single injection of different doses of RPCH showed no significant change of molt durations compared to the control (Table 2). Similarly, six injections of the peptides also showed no significant difference of molt duration indicating that the synthetic RPCH did not affect the molting cycle of the shrimp.

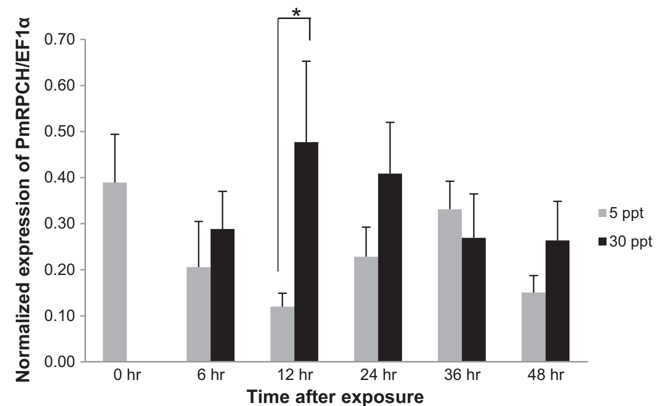

Fig. 3. Changes in *PmRPCH* mRNA levels under hypersalinity stress. Shrimp were acclimatized in 5 ppt seawater for one week before rearing in 30 ppt seawater (dark bars) or continuing rearing in 5 ppt seawater (gray bars). *PmRPCH* copy numbers normalized to that of *EF-1α* were determined by qRT-PCR at 6, 12, 24, 36 and 48 h. Values are shown as means ± SEM (n = 5). An asterisk depicts significant difference at $p < 0.05$ by two-way ANOVA and Bonferroni's test.

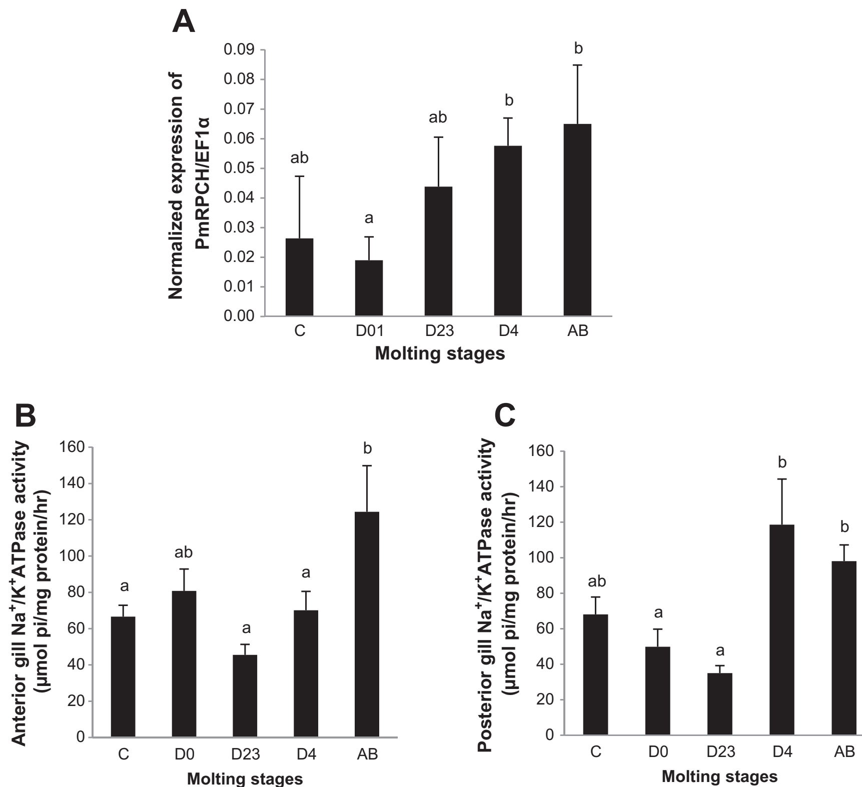

Fig. 4. Expression profile of eyestalk *PmRPCH* mRNA and gill Na^+/K^+ ATPase activity during the molting cycle. (A) *PmRPCH* mRNA expression in each stage of molt was determined by reverse transcription real-time PCR. The relative expression of *PmRPCH* compared to that of *EF-1α* in the early pre-molt stage was used as a calibrator for the relative expression values of *PmRPCH* in the other molting stages. Values are shown as means ± SEM (n = 6). Both anterior (B) and posterior (C) gill Na^+/K^+ ATPase activities were determined during molting cycle. The alphabets represent significant difference ($p < 0.05$) analyzed by one way ANOVA and Duncan's test. Molting stages are depicted as follows: intermolt (C); early pre-molt (D_0); mid pre-molt (D_{23}); late pre-molt (D_4) and post-molt (AB).

4. Discussion

RPCH is a crustacean neuropeptide that is structurally related to the adipokinetic hormone (AKH) in insects. Peptides in the AKH/RPCH family generally contain 8–10 amino acid residues of the mature peptide with common features including modification at the N- and C-termini by pyroglutamate and amidation, respectively. All crustacean RPCHs identified to date share identical mature peptide sequences. The fourth and eighth positions of their mature peptides usually contain aromatic amino acids Phe and Trp, respectively (Gäde, 2009). The deduced amino acid sequence of the mature PmRPCH obtained in this study shared all the aforementioned features of the RPCH peptides. Comparison of amino acid sequences revealed variations at three positions between mature RPCHs (Asn^3, Pro^6 and Gly^7) and AKHs (Thr^3, Thr^6 and Ser^7 or Asp^7) as shown in Fig. 1. Whereas the insect AKHs were known to be functionally involved in energy metabolism e.g. activation of glycogen phosphorylation and lipase enzymes in the fat body for energy supply, especially in the flight muscles (Gade, 2004), crustacean RPCHs primarily function in the aggregation of red pigment granules in response to environment (Rao, 2001). However, it has been demonstrated that the insect AKH could elicit pigment-concentration in prawn (Mordue and Stone, 1977). In addition, the synthetic crustacean RPCH (Panbo-RPCH) was able to trigger lipid mobilization in stinkbug, *Nezara viridula* (Gäde et al., 2003). The cross-activity between these two hormones was correlated to the similarity in their structures. In addition, this also implies that different functional activities of RPCH/AKH may require the binding of the peptides to different receptors presented in crustaceans and insects. Interestingly, Marco and Gäde (2010) recently reported that the Lue^2 amino acid residue was essential for the chromatophorotropic activity of RPCH since the change from Lue^2 to Val^2 resulted in a significant drop in its activity (Marco and Gäde, 2010). Further studies on the structure–function relationship as well as the ligand-receptor binding of RPCH/AKH are required for understanding detailed mechanisms of action of each peptide.

Immunohistochemical studies in several species revealed the presence of the RPCH peptide in crustacean central nervous system (CNS) e.g. optic ganglia, brain, thoracic ganglia and abdominal nerve cord (Mangerich et al., 1986; Nusbaum and Marder, 1988; Klein et al., 1995; Chung and Webster, 2004). Recent study by Kornthong et al. also confirmed the expression of RPCH in the CNS of the mud crab *Scylla olivacea* by RT-PCR and in situ hybridization (Kornthong et al., 2013). Our result showed that *P. monodon RPCH* mRNA was markedly expressed in the eyestalk and other nervous tissues such as the brain, thoracic ganglia and abdominal nerve cord (Fig. 2), thus conforming to the expression of RPCH in other crustaceans.

Besides its primary function in pigment aggregation, previous studies have demonstrated the metabolic functions of RPCH in both crustaceans and insects. For instance, injection of a synthetic RPCH increased carbohydrate, especially glucose in the hemolymph of the isopod *P. scaber* (Zralá et al., 2010). The role in osmoregulation of PmRPCH was determined under hypersalinity change in this study. The eyestalk *PmRPCH* mRNA levels were transiently increased to 3 folds after the shrimp that had been acclimated in 5 ppt seawater were transferred to 30 ppt salinity for 12 h compared with the control shrimp maintained in 5 ppt seawater (Fig. 3). This short time response of *PmRPCH* expression might involve rapid activation of the osmoregulatory system. In *P. monodon* and *Macrobrachium rosenbergii*, hemolymph osmolality was raised under hyper-osmotic condition (Wilder et al., 1998; Lin et al., 2000; Tantulo and Fotedar, 2007). Moreover, activity of ion transport enzymes, particularly Na^+/K^+ ATPase in gills rapidly increased within 3 h after exposing to hypersaline water in a teleost, *Fundulus heteroclitus* (Mancera and McCormick, 2000). However, in *P. monodon*

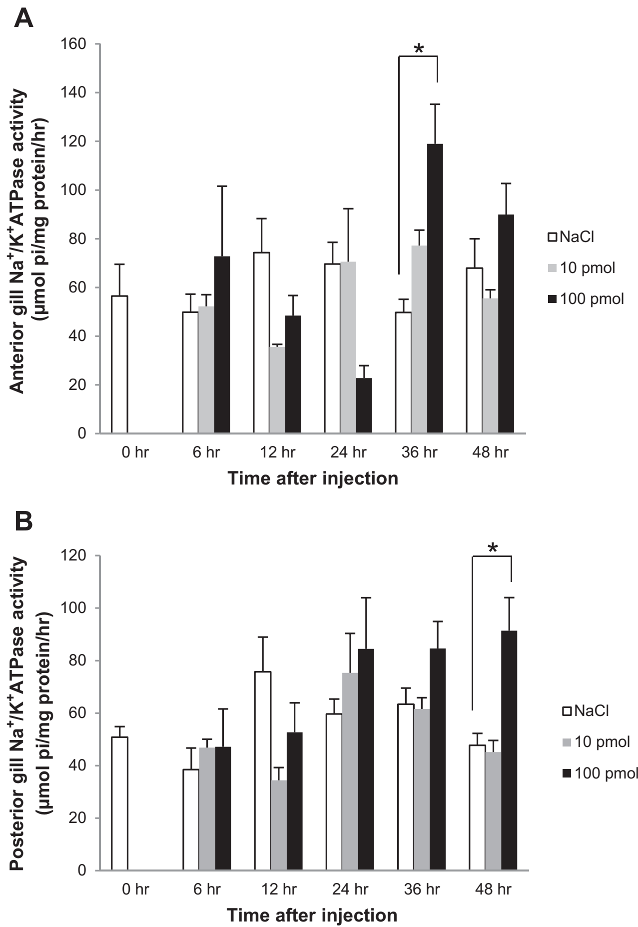

Fig. 5. Effect of RPCH peptide on gill Na^+/K^+ ATPase activity. Both anterior (A) and posterior (B) gill Na^+/K^+ ATPase activities were determined after injection of 10 pmol, 100 pmol RPCH or NaCl at different time courses. Values are shown as mean ± SEM (n = 5). An asterisk represents significant difference at $p < 0.05$ analyzed by two-way ANOVA and Bonferroni's test.

the activity of gill Na^+/K^+ ATPase measured after the shrimp were subjected to salinity changes for one week did not change comparing with the activity at the beginning (Buranajitpirom et al., 2010). Although there was no evidence about the ion transport function of RPCH at present, previous study demonstrated that RPCH could exert an overlapping function with an eyestalk's crustacean hyperglycemic hormone (CHH) in *P. scaber* (Zralá et al., 2010). As CHH belongs to the same eyestalk neuropeptide family as an ion transport peptide (ITP) (Audsley et al., 1992), our result therefore suggests that PmRPCH probably functions as an ion transport peptide, an eyestalk hormone not yet identified in *P. monodon*. However, direct evidence is needed to confirm the ion transport function of PmRPCH.

Molting is an important process required for growth in crustaceans. It involves discarding of an old exoskeleton and replacing with a new one. Dramatic changes in several physiological mechanisms, especially osmoregulation occur during the molting process (Chang, 1995). Molting is known to be regulated mainly by a molt-inhibiting hormone (MIH), an eyestalk neuropeptide that inhibits the synthesis and the release of ecdysteroids or molting hormone from the Y-organ (Chang and Mykles, 2011). In addition, other eyestalk peptides such as CHH and mandibular organ-inhibiting hormone (MO-IH) were shown to play a role in ecdysteroid synthesis (Chung, 2010). As mentioned earlier that RPCH could have overlapping functions with other eyestalk's peptides, possible involvement of PmRPCH in regulating the molting process in *P. monodon* was therefore investigated. Our result showed that injection of the synthetic RPCH did not affect the molt duration of the shrimp either by single injection or multiple injections (Table 2). Therefore it is possible that PmRPCH was not directly required for triggering or mediating the molting process. In addition, the expression levels of *PmRPCH* mRNA in shrimp eyestalk during each stage of the molting cycle were determined. A dramatic increase of *PmRPCH* mRNA expression was obviously observed from its lowest level in the early pre-molt stage (D_0) up to the maximum level at the late pre-molt stage (D_4). In the pre-molt stage, osmoregulation, one of the molting processes, was changed in crustaceans. For example, hemolymph osmolality in *Litopenaeus vannamei*, *M. rosenbergii* and *Farfantepenaeus duorarum* was increased in the late-pre-molt and molt stages (Galindo et al., 2009; Wilder et al., 2009; Bonilla-Gómez et al., 2012). Gill Na^+/K^+ ATPase and carbonic anhydrase activities were raised in the pre-molt stages in *Macrobrachium nipponense* and *L. vannamei* (Wang et al., 2003; Jasmani et al., 2010). Similarly, our result showed that gill Na^+/K^+ ATPase activity in *P. monodon* (Fig. 4 A and B) was increased in the late pre-molt through molting stages. In addition, we also demonstrated that injection of synthetic RPCH peptide stimulated Na^+/K^+ ATPase activity in the anterior and posterior gills at 36 h and 48 h after injection, respectively (Fig. 5A and B). Since RPCH signaling transduction raised intracellular Ca^{2+} by activating some voltage-gated Ca^{2+} channels (Milograna et al., 2010), it is possible that following RPCH injection, the homeostasis of Ca^{2+} was regulated through a Na^+/Ca^{2+} exchanger (Carafoli, 2002) resulting in a Na^+ influx that activated the Na^+/K^+ ATPase activity in the gills. Further study to determine the actual Na^+ flux in the shrimp is needed to explain the mechanism of action for this effect of RPCH injection. Taken together, our results provide supportive evidences for a role of PmRPCH in osmoregulatory responses during the molting process.

In summary, our current study identified and characterized a cDNA encoding RPCH in *P. monodon*. Determination of *PmRPCH* mRNA expression levels reveals that *PmRPCH* expression was transiently elevated upon hypersalinity change suggesting the role of PmRPCH in osmoregulation. Although the synthetic RPCH did not exhibit any influence on the molt duration of the shrimp, it could stimulate the activity of gill Na^+/K^+ ATPase. Our result further demonstrates that gill Na^+/K^+ ATPase activity significantly increased during the pre-molt stages. Hence, we propose that PmRPCH may not directly trigger the molting process, but is probably required for regulating hemolymph osmolality and ion transport enzymes during the molt.

Table 2
Duration of molt after synthetic RPCH injection.

Injection	Group (number of shrimp)	Duration of molt (days; mean ± SD)
Single injection	Control (n = 7)	10.5 ± 0.98
	1 pmol RPCH (n = 7)	11.2 ± 1.11
	10 pmol RPCH (n = 7)	10.8 ± 1.57
	100 pmol RPCH (n = 7)	10.5 ± 1.13
6 injections	Control (n = 10)	11.7 ± 1.33
	100 pmol RPCH (n = 9)	12.0 ± 1.11

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