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SHORT COMMUNICATION

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Expression and Purification of Recombinant MrRPCH from Eyestalk of the Giant Freshwater Prawn (*Macrobrachium rosenbergii*) for Disease Resistance and Growth Promotion in Crustaceans

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ABSTRACT

Growth regulation in the giant freshwater prawn (*Macrobrachium rosenbergii*) is closely linked to neuropeptides such as red pigment concentrating hormone (MrRPCH), a single-chain polypeptide structurally related to proinsulin. In this study, the MrRPCH gene was cloned into a modified pRSET_A expression vector and transformed into *Escherichia coli* BL21 trxB (DE3) cells. Upon IPTG induction, the recombinant MrRPCH protein (~12kDa) was expressed predominantly in inclusion bodies. The protein was successfully purified using the MagneHis™ Protein Purification System, and its purity was confirmed by SDS-PAGE analysis. This study provides a reliable method for high-yield expression and purification of MrRPCH, paving the way for future applications in aquaculture, including disease resistance enhancement and growth promotion in crustaceans.

Keywords: Disease resistance, Growth hormone, MrRPCH, *Macrobrachium rosenbergii*, Recombinant protein.

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INTRODUCTION

Crustacean neuroendocrine regulation is primarily mediated by neuropeptides secreted from the X-organ–sinus gland (XO–SG) complex in the eyestalk, which orchestrate various physiological processes including molting, reproduction, metabolism, and osmoregulation (Chung & Webster, 2005; Tsutsui et al., 2016). Among these, the crustacean hyperglycemic hormone (CHH) family including CHH, molt-inhibiting hormone (MIH), gonad-inhibiting hormone (GIH) and red pigment concentrating hormone (RPCH) plays a pivotal role in these regulatory mechanisms (Katayama et al., 2004; Marco et al., 2017).

CHH family peptides typically consist of 72–78 amino acids with six conserved cysteine residues forming three disulfide bridges, essential for their structural integrity and biological activity (Tsutsui et al., 2016). RPCH, a smaller peptide compared to CHH, was initially identified for its role in pigment aggregation in chromatophores but has since been implicated in regulating growth, cellular differentiation, reproduction, and biosynthesis (Marco et al., 2017; Wei et al., 2021).

In *Macrobrachium rosenbergii*, the giant freshwater prawn, MrRPCH-like hormones have been identified and are believed to function as growth factors, influencing somatic growth and reproductive maturation (Yang et al., 2020). The expression of RPCH and its receptor in this species suggests a complex regulatory network affecting various physiological processes (Marco et al., 2017).

Recombinant production of neuropeptides like MrRPCH in *Escherichia coli* has been challenging due to issues such as low yield, misfolded proteins, and the formation of inclusion bodies (Zweng et al., 2023). *E. coli* BL21 trxB (DE3), a strain engineered to enhance disulfide bond formation in the cytoplasm, making it suitable for expressing eukaryotic proteins with multiple cysteine residues (Singh et al., 2023). The BL21 trxB strain lacks thioredoxin reductase and glutathione reductase, promoting an oxidizing intracellular environment conducive to proper protein folding of disulfide-rich proteins such as neuropeptides (Singh et al., 2023). Advancements in molecular cloning and expression systems, including the use of specialized *E. coli* strains like BL21 trxB (DE3) and vectors such as pRSET_A,

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have improved the production of disulfide-rich proteins (Teufel et al., 2021). These systems facilitate proper protein folding and enable efficient purification using affinity chromatography techniques (Teufel et al., 2021).

The present study aims to clone the MrRPCH gene from the eyestalk sinus gland of adult *M. rosenbergii*, express it in *E. coli* BL21 trxB (DE3) cells, and purify the recombinant protein using a histidine-tag affinity system. This approach will provide insights into the physiological role of MrRPCH and assess its potential application as a growth-promoting or antibacterial agent in aquaculture.

MATERIALS & METHODS

Bacterial Strain and Vector Construction

The bacterial expression system employed in this study was *E. coli* BL21 trxB (DE3), a strain engineered to enhance disulfide bond formation in the cytoplasm.

Total RNA was extracted from the eyestalk sinus gland of adult *M. rosenbergii* using Trizol® reagent (Invitrogen, USA) according to the standard protocol described by Chomczynski & Sacchi (1987). First-strand cDNA was synthesized using oligo(dT) primers according to established methods (Frohman et al., 1988; Sambrook & Russell, 2001). A partial cDNA fragment corresponding to the red pigment concentrating hormone (MrRPCH) was previously isolated from a subtractive library. PCR amplification of the full-length MrRPCH coding region was performed using gene-specific primers: RPCH-F (5′-ATGGTTCGCAGTGGAGTTTCC-3′) and RPCH-R (5′-GGCCAGGTATTCTTCGTCTTG-3′), which were designed based on previously reported neuropeptide sequences of crustaceans (Udomkit et al., 2004; Yang et al., 2020). The primers were designed to include restriction sites for *BamHI* and *EcoRI* to facilitate directional cloning.

The ~800bp PCR product was purified using a gel extraction kit (Qiagen, Germany), digested with *BamHI* and *EcoRI* and ligated into a pRSET_A vector (Life Technologies), which provides a strong T7 promoter, a 6×His-tag at the N-terminus and a bacterial ribosome binding site. The recombinant construct, designated MrRPCH_pRSET_A, was transformed into competent *E. coli* DH5α for plasmid propagation and sequence confirmation via Sanger sequencing (Macrogen, Korea). Verified plasmids were then introduced into *E. coli* BL21 trxB (DE3) by heat shock at 42°C for 45s followed by selection on LB agar plates containing 100μg/mL ampicillin (Zweng et al., 2023).

Expression Induction

A single colony of recombinant *E. coli* was inoculated into 5mL of 2×YT medium containing 100μg/mL ampicillin and grown overnight at 37°C with shaking at 200rpm. The overnight culture was diluted 1:100 into fresh 200mL 2×YT medium and incubated at 37°C until reaching an OD600 of 0.4–0.6. To induce recombinant protein expression, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4mM and the culture was further incubated for 4 hours under the same conditions (Teufel et al., 2021).

Cells were harvested by centrifugation at 4,000×g for 20min at 4°C, and the bacterial pellet was either processed immediately or stored at −20°C. The growth curve was monitored, and samples were collected at different time points post-induction to assess protein expression levels (Zweng et al., 2023).

Purification Protocol

Bacterial pellets were resuspended in 50mM Tris-HCl (pH 8.0) containing 100μg/mL lysozyme and incubated on ice for 30min. To facilitate lysis, the suspension underwent three cycles of freeze-thaw using liquid nitrogen and a 37°C water bath. Following the final thaw, 20μg/mL DNase I and 20mM $MgSO_4$ were added, and the suspension was incubated at 37°C for 30min to reduce viscosity caused by genomic DNA.

Cell debris was removed by centrifugation at 20,000×g for 30min at 4°C, and the resulting pellet, containing inclusion bodies, was washed twice with PBS (pH 7.4) and once with buffer containing 2M urea to remove loosely associated contaminants. The inclusion bodies were then solubilized in buffer containing 6 M guanidine hydrochloride, 200mM NaCl, 100mM Tris-HCl, and 1mM EDTA (pH 8.3) supplemented with 10mM β-mercaptoethanol. The solution was gently agitated overnight at 4°C to ensure complete solubilization and denaturation of proteins (Singh et al., 2023).

The solubilized protein was clarified by centrifugation and purified using the MagneHis™ Ni^{2+}-NTA magnetic bead system (Promega, USA). Binding was carried out under denaturing conditions, and after extensive washing, the recombinant His-tagged MrRPCH protein was eluted with buffer containing 250mM imidazole. Protein refolding was achieved via stepwise dialysis against decreasing concentrations of urea (4M, 2M, 1M, and 0M) in Tris-buffered saline (TBS), each for 6 hours at 4°C (Teufel et al., 2021).

Protein Analysis

The purity and molecular mass of the recombinant MrRPCH were assessed using SDS–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Protein samples were mixed with Laemmli buffer, boiled for 5min, and loaded onto 15% acrylamide/bis-acrylamide gels. Electrophoresis was performed at 100V for 90min. After separation, the gels were stained with Coomassie Brilliant Blue R-250 and destained using a methanol/acetic acid solution to visualize the protein bands (Zweng et al., 2023).

The molecular weight of the expressed MrRPCH protein was approximately 12kDa, consistent with the predicted size including the His-tag. Densitometric analysis of the bands was conducted using ImageJ software to estimate protein purity and relative yield. In future studies, western blotting with anti-His antibodies or mass spectrometry could be employed for further verification (Teufel et al., 2021).

RESULTS AND DISCUSSION

Vector Construction and Protein Expression

The MrRPCH gene was successfully inserted into the

pRSET_A vector, with cloning accuracy verified through restriction enzyme digestion and DNA sequencing. The construct included a full-length open reading frame (ORF) of MrRPCH, flanked by *BamHI* and *EcoRI* restriction sites, and was situated downstream of a T7 promoter and an N-terminal His-tag sequence (Fig. 1). Electrophoresis of the digested plasmid displayed a clear 800bp fragment, consistent with the target gene length, and DNA sequencing confirmed proper orientation without mutations.

Following transformation into *E. coli* BL21 trxB (DE3), recombinant protein expression was induced using 0.4mM IPTG. SDS–PAGE analysis revealed a distinct ~12kDa protein band corresponding to the expected size of the recombinant MrRPCH, inclusive of the His-tag. No protein band was detected in uninduced controls, confirming controlled expression under the T7 promoter. Notably, the recombinant protein localized predominantly in the insoluble fraction, forming inclusion bodies a common phenomenon in prokaryotic expression of disulfide-bonded eukaryotic proteins (Villaverde & Carrió, 2003).

The trxB mutation in *E. coli* promotes disulfide bond formation by disrupting the thioredoxin reductase system, enabling oxidative folding in the cytoplasm (Berkmen, 2012). However, overexpression likely exceeded the folding capacity of the cell, leading to misfolding and aggregation. This behavior is consistent with other studies on crustacean peptides such as CHH and RPCH, where expression in *E. coli* often results in inclusion body formation (Udomkit et al., 2004; Rosano & Ceccarelli, 2014).

Protein Purification and Molecular Confirmation

The insoluble recombinant MrRPCH protein was purified from inclusion bodies using guanidine hydrochloride as a denaturant. The presence of 10mM beta-mercaptoethanol ensured reduction of disulfide bonds during solubilization. Purification using the MagneHis™ Ni^{2+}-NTA system enabled high-affinity binding of the His-tagged protein and effective elution with imidazole.

SDS–PAGE analysis post-purification confirmed a single strong band at ~12kDa, and densitometric quantification estimated purity at >90% (Fig. 2). This level of purity is acceptable for downstream bioassays or antibody generation (Rosano & Ceccarelli, 2014). To obtain bioactive protein, stepwise dialysis against decreasing concentrations of urea was conducted for protein refolding. Although in vitro functional assays were not performed, past research demonstrates that properly refolded disulfide-containing hormones like CHH regain biological activity (Tsumoto et al., 2003; Chong et al., 2019).

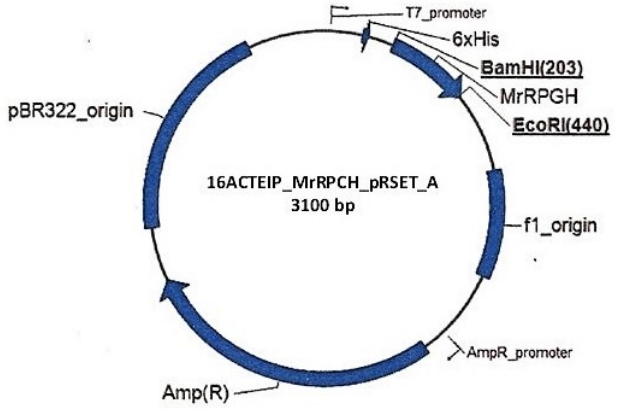

Fig. 1: Schematic map and partial nucleotide sequence of the recombinant expression plasmid MrRPCH_pRSET_A vector (3100bp). The sequence includes a T7 promoter, 6×His tag, Xpress™ epitope, and multiple cloning site with *BamHI* and *EcoRI* flanking the MrRPCH insert.

T7 promoter — RBS

21 AATACGACTC ACTATAGGGA GACCACAACG GTTTCCCTCT AGAAATAATT TTGTTTAACT TTAAGAAGGA

Polyhistidine (6xHis) region

91 GATATACAT **ATG** CGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT AGC ATG ACT
Met Arg Gly Ser His His His His His His Gly Met Ala Ser Met Thr

T7 gene 10 leader — Xpress™ Epitope — *Bam*H I

148 GGT GGA CAG CAA ATG GGT CGG GAT CTG TAC GAC GAT GAC GAT AAG GAT CGA TGG GGA
Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp Asp Asp Asp Lys Asp Arg Trp Gly

EK recognition site — EK cleavage site

Xho I *Sac* I *Bgl* II — *Pst* I *Pvu* II — *Kpn* I *Nco* I — *Eco*R I — *Bst*B I — *Hind* III

205 TCC GAG CTC GAG ATC TGC AGC TGG TAC CAT GGA ATT CGA AGC TTG ATC CGG CTG CTA
Ser Glu Leu Glu Ile Cys Ser Trp Tyr His Gly ile Arg Ser Leu Ile Arg Leu Leu

T7 reverse priming site

262 ACA AAG CCC GAA AGG AAG CTG AGT TGG CTG CTG CCA CCG CTG AGC AAT AAC TAG CAT
Thr Lys Pro Glu Arg Lys Leu Ser Trp Leu Leu Pro Pro Leu Ser Asn Asn *** His

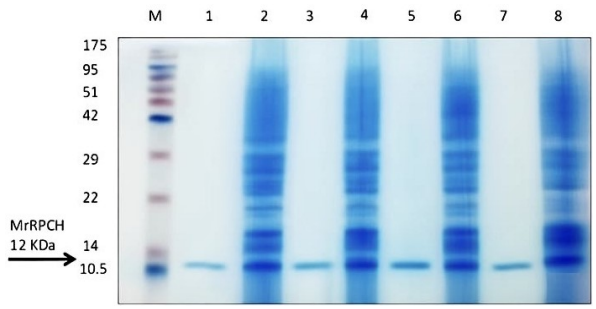

Fig. 2: SDS-PAGE analysis of recombinant MrRPCH expression in *E. coli* BL21. Lane M = molecular weight marker; Lanes 1,3 = supernatant after IPTG induction; Lanes 2,4 = pellet after IPTG induction; Lanes 5,7 = supernatant without IPTG; Lanes 6,8 = pellet without IPTG. The ~12kDa MrRPCH protein band is indicated.

Implications and Future Applications

The availability of purified recombinant MrRPCH enables advanced studies into its biological role in *Macrobrachium rosenbergii*. As a member of the CHH family, RPCH is involved in regulating pigment concentration, growth, reproduction, and osmoregulation (Marco et al., 2017; Wei et al., 2021). Recombinant MrRPCH can be used for physiological studies, including hormone injections, RNA interference (RNAi), and immunodetection using specific antibodies.

In aquaculture, recombinant peptide hormones have been used to influence sex differentiation, stress tolerance, and immune modulation. Recombinant insulin-like androgenic gland hormone (IAG) and CHH have successfully promoted desired traits in shrimp (Treerattrakool et al., 2008; Ventura et al., 2012). MrRPCH has the potential to serve as a biostimulant or additive in formulated diets, potentially enhancing growth rates or disease resistance in prawns.

Furthermore, MrRPCH could serve as an immunogen to develop monoclonal or polyclonal antibodies for hormone tracking. These assays would allow researchers to study hormone regulation under different physiological or environmental conditions. Future work could also use MrRPCH in transcriptomic or proteomic studies, examining its impact on downstream effectors.

The recombinant expression strategy outlined here may also be adapted for other difficult-to-express neuropeptides such as molt-inhibiting hormone (MIH), gonad-inhibiting hormone (GIH), and vitellogenesis-inhibiting hormone (VIH), expanding tools for crustacean functional genomics. The integration of such hormonal tools with CRISPR/Cas9 or RNAi could drive new breakthroughs in crustacean endocrinology and aquaculture biotechnology (Yang et al., 2020; Zweng et al., 2023).

Conclusion

This study demonstrated the successful cloning, expression, and purification of recombinant red pigment concentrating hormone (MrRPCH) from the giant freshwater prawn (*Macrobrachium rosenbergii*) using an *E. coli* BL21 trxB (DE3) system. The recombinant protein (~12 kDa) was expressed predominantly as inclusion bodies, purified under denaturing conditions, and refolded to obtain a highly pure preparation. The established protocol provides a reproducible method for producing MrRPCH and may be applied to other disulfide-rich crustacean neuropeptides. Availability of recombinant MrRPCH offers a useful tool for functional studies, including bioassays, immunological detection, and molecular analyses of crustacean growth and stress regulation. Moreover, it holds potential for aquaculture applications, such as improving growth performance or immune responses in prawns. Further in vivo validation of its biological activity will be essential to support its practical use.

DECLARATIONS

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Conflict of Interest: The authors state that there are no conflicts of interest with this study.

Data Availability: All data generated or analysed during this study are available from the corresponding author upon reasonable request.

Ethics Statement: This study involved the collection of eyestalk tissues from *Macrobrachium rosenbergii*, and all procedures were conducted in accordance with institutional ethical guidelines. Approval was granted by the Research and Development Institute Committee, Kalasin University, Thailand (Approval No. KSU-AE-036).

Author's Contribution: Keeravit Petjul: Conceptualization, Methodology, Writing - original draft. Nattapon Kan-a-roon: Data curation, Formal analysis. Prasit Khunsanit: Investigation, Resources. Urai Kollboon: Supervision, Validation. Tanaphoom Boonmee: Visualization, Writing - review & editing.

Generative AI Statement: The authors declare that no Gen AI/DeepSeek was used in the writing/creation of this manuscript.

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