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Thioredoxin fused CHH1 protein as antigen for polyclonal antisera: Application to regulate glycemia in *Penaeus monodon*

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Crustacean hyperglycemic hormone (CHH) family neuropeptides have been in research limelight for the past two decades due to their importance in the regulation of glycemia, moulting and gonad development in crustaceans. Under natural conditions, low levels of CHH neuropeptide and the structural similarity of the three CHH family neuropeptides limit their purification directly from the animal. In this study, we isolated the mature region of the CHH1 gene, constructed the recombinant translation expression vector (pET32a+ - PmCHH1) and produced thioredoxin fused protein in *Escherichia coli* (BL21 (DE3) pLysS). The translation expression vector construct (pET32a+ - PmCHH1) was successfully built for production of thioredoxin fused mature CHH1 protein (mf-PmCHH1-29.47 kDa). Mf-PmCHH1 produced a hyperglycemic effect similar to that of the eyestalk extract when experimentally injected into adult eyestalk ablated *Penaeus monodon*. The polyclonal antiserum (anti-mf-PmCHH1) was developed in mice against the purified thioredoxin fused mf-PmCHH1 protein. A hypoglycemic effect was induced in adult *P. monodon* by the polyclonal antiserum which was raised against thioredoxin fused CHH1 protein. Immunolocalization of CHH1 producing neurosecretory cells in the eyestalk of *P. monodon* was a practical result obtained with the polyclonal antiserum anti-mf-PmCHH1. Therefore, mf-PmCHH1 and its antiserum (anti-mf-PmCHH1) are added to the list of tools to better understand the endocrine mechanisms regulating glycemia and reproduction in *P. monodon*.

Keywords: CHH neuropeptides, Crustacean hyperglycemic hormone, Diabetogenic factor, Immunolocalization, Recombinant/fusion/chimeric protein, Shrimps

The exclusive association of crustacean hyperglycemic hormone (CHH) family controlling the crustacean metabolism, osmoregulation, moulting and reproduction account for a number of physiological effects known for past two decades. The eyestalk factors regulating blood glucose concentrations in blue crab, *Callinectes sapidus* serve as a potent hyperglycemia inducing regulator. This hyperglycemia factor is termed as "diabetogenic factor"[1]. The CHH plays a major role in controlling glucose levels in the haemolymp and is highly concentrated in the sinus gland of the eyestalk[2]. The CHH is classified as type I peptide of the CHH family neurohormones based on the absence of glycine residue at position 12 in the mature peptide[3]. The isolation, characterization, localization, secretory dynamics and *in silico* analysis of CHH from several crustaceans has been investigated and the process continues to contribute to the knowledge concerning the crustacean hyperglycemia neuropeptide as one of the major hormones regulating osmoregulation, growth and reproduction[4-9]. The difficulty in isolating CHH from natural sources due to the miniscule presence in the animal body and the similarity in structure of the CHH family neurohormones renders the platform of recombinant protein expression useful and highly efficient.

The recombinant protein of CHH1 expressed in *Pichia pastoris* demonstrated its ability to elevate glucose level in haemolymph[10]. Further, two more isoforms of CHH cDNAs (CHH2 and CHH3) have also been isolated from *P. monodon* and the biological activities of the recombinant proteins expressed in *P. pastoris* were determined[11]. Recombinant CHH family hormone has been expressed in bacterial system and proven to be biologically active[4,12,13]. In addition, a CHH1 recombinant protein (Pem-CHH1) has been developed in pET32a+ system, and the specific antibody raised against Pem-CHH1 shown to impair the hyperglycemia conditions in *P. monodon*[14].

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Further, in the light of fusion (or chimeric) proteins being utilized in protein science research for immunodetection, protein therapies, vaccine development, functional genomics, analysis of protein trafficking and protein-protein interactions[15,16], the approach of thioredoxin-fused mature CHH1 protein was designed. Here, in this study, we focused on (i) development and production of thioredoxin fused mature CHH1 protein of *P. monodon* in a *E. coli* expression system (*E. coli* BL21-DE3-pLysS); and (ii) use of thioredoxin-fused mature CHH1 antigen to raise polyclonal antisera and thereby demonstrate ELISA as a tool for measuring circulating CHH1 and immunolocalization of CHH1 producing neuro-endocrine cells in the eyestalk of *P. monodon*. The possible *in vivo* effects of thioredoxin fused mature CHH1 protein and polyclonal antiserum in the process of glycemia regulation in *P.monodon* have also been demonstrated.

Material and Methods

Experimental animals

Penaeus monodon (10-15 g) obtained from the local hatchery were stocked and maintained in a laboratory recirculating aquaculture system (RAS) for shrimps integrated with nitrifying bioreactor in sea water having 15 g L^{-1} salinity. Water quality was maintained at pH 6.8-7.8; total ammonia 0.037 ± 0.047; nitrite 0.58 ± 0.3; total alkalinity ($CaCO_3$) 97.5 ± 18.03; and total hardness 5500 ± 360.6 mg L^{-1}. The animals were fed on commercially available pelleted feed (Higashimaru, India) containing 53% protein, 9% fat, 3% fiber, 2% calcium, 1% phosphorus, 20% ash and 12% moisture.

RT-PCR amplification and TA cloning in pGEM-T easy vector

Total RNA was extracted using TriReagent (Sigma, USA) from the eyestalk of *P. monodon*. The total RNA was subjected to DNase treatment with RNase free DNase 1 (New England Biolabs, UK) by adding 0.2 U of enzyme to 1 µg RNA and incubated at 37°C for 10 min followed by inactivation at 75°C for 10 min. RNA concentration and quality were determined by absorbance (Abs 260/280 nm) measurement using a UV-Visible Spectrophotometer (Hitachi, Japan). An aliquot of 5 µg RNA was reverse transcribed using reagents of New England Biolabs, USA. The 20 µL reaction mix contained M-MuLV reverse transcriptase (200 U), RNase inhibitor (8 U), Oligo $(dT)_{12}$ primer (40 pmoles), dNTP mix (1 mM), RTase buffer (1X) and $MgCl_2$ (2 mM), and the cDNA was synthesized by incubating the reaction mix at 42°C for 1 h followed by inactivation at 65°C for 20 min[17].

Gene specific primers were designed to amplify the complete coding sequence (CDS) of CHH1 based on the GenBank sequence of CHH1 (AF233295) of *P. monodon* in the NCBI database. PCR was conducted in a 25 µL reaction volume containing 2.5 µL 10X buffer, 2.5 mM dNTP (2.5 µL), 10 pmol μL^{-1} (1.0 µL) forward (5'-CCTGGAAGTTGCTGACC GTCGCTC-3') and reverse primers (5' CTTGCCGAG CCTCTGTAGGGCGG-3'), 0.5U μL^{-1} (1.0 µL) *Taq DNA* polymerase (NEB, USA) and 1 µL cDNA template. The PCR was carried out in an Eppendorf Thermal cycler (Germany) with an initial denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 40 s; annealing 60°C for 45 s; and extension at 72°C for 40 s. The amplified PCR products were resolved by 1% agarose gel electrophoresis[17]. The PCR product of CHH1 was cloned in to pGEM-T easy vector (Promega, USA) by following the manufacturer's protocols and the nucleotide sequence was determined following the method of ABI ™ Prism Dye termination cycle at SciGenom, Kochi.

Construction of translation expression vector

The amino acid sequence of CHH1 (Accession GQ221085) was analyzed using SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/)[18]. The mature region was identified and the primers with restriction sites were designed. The forward primer (CHH1-F- 5'-*GAATTC*AGCCTATCCTTCAGGTCTTGCACGGGC-3') contained *EcoR* I site (italics) and 29 nucleotide residues encoding the N terminal amino acid residues of the mature CHH1 of *P.monodon*. The reverse primer (5'-*CTCGAG*CTTGCCGAGCCTCTGTAGGGCGG-3') contained *Xho* I site (italics) and 19 nucleotide residues encoding the C-terminal amino acid residues of mature CHH1. The *Pfu* DNA polymerase amplified PCR product was subcloned into pJET 1.2 blunt end vector (Fermentas, USA) and the nucleotide sequence was confirmed. Subsequently, the inserts were released from pJET 1.2 by *EcoR* I and *Xho* I digestion and ligated into the *EcoR* I/*Xho* I site of the expression plasmid pET-32a+ (Novagen, USA). The expression plasmid was designated as mf-PmCHH1+pET32a+ (mf-PmCHH1: Mature fusion-*Penaeus monodon* CHH1).

Expression of mature CHH1 fusion protein using Pm-CHH1+ pET32a+ constructs

For recombinant expression of mature CHH1, *Escherichia coli* BL21 (DE3) pLysS was used. This

strain has the advantage of being deficient in both the lon and ompT proteases and harbours the T7 bacteriophage RNA polymerase gene, which permits the specific expression of heterologous genes driven by the T7 promoter (19-21). *E. coli* BL21 (DE3) pLysS competent cells (Novagen, USA) were transformed with mf-PmCHH1+pET32a+ and the transformants were selected on LB/ampicillin (100 µg μL^{-1}) plates. Bacterial cells from single colonies were grown in 3 mL LB/ampicillin (100 µg μL^{-1}) medium and incubated at 37°C at 250 rpm until the Abs_{600} of 0.5 was obtained. Overnight incubation was avoided to reduce the over expression of beta lactamase protein. As controls, *E. coli* BL21 (DE3) pLysS without vector and *E. coli* BL21 (DE3) pLysS with pET32a+ vector were also processed. The entire 3 mL culture was added to 100 mL LB/ampicillin (100 µg μL^{-1}) medium and further incubated at 37°C for 2 h until Abs_{600} of between 0.5-0.8 was attained (0.6 Optimum Abs for protein expression). Thereafter, IPTG was added to a concentration of 1 mM to promote protein production. The mixture was incubated for an additional 3.5-4 h after which bacterial cells were harvested by centrifugation. The cells were subjected to breakage by sonication using two lysis buffers, namely buffer 1:(50 mM KH_2PO_4-pH 7.8, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 10 mM imidazole) and buffer 2:(31.25 mM Tris buffer pH 6.8, 25% Glycerol, 10% SDS) to separate the soluble and the insoluble fractions. The fractions were subjected to 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The negative controls included induced *E. coli* BL21 (DE3) pLysS and *E. coli* BL21 (DE3) pLysS cells transformed with pET32a+ lacking any insert. The fractions of the controls were prepared by the same method described for induced CHH1 and subjected to SDS-PAGE [17].

SDS-PAGE purification, refolding, and Western blot of the mature fusion Pm-CHH1 protein

In order to exclude the non-specific protein bands and the denaturants such as urea in the Ni-NTA purified recombinant samples, they were subjected to polyacrylamide gel purification. The mature Pm-CHH1 fusion protein (mf-PmCHH1) was run on 12% SDS-PAGE and the bands of mf-PmCHH1 fusion protein were extricated from the unstained portion of the gel comparing to the stained portion. An aliquot of 1 mL extraction buffer (50 mM Tris-Cl pH8, 0.1 mM EDTA and 0.15 M NaCl), was added to the gel pieces and homogenized using a glass homogenizer. The extraction buffer was added to immerse the gel pieces completely. The homogenized recombinant protein was incubated at 30°C overnight with shaking at 200-250 rpm. The samples were centrifuged at 5000-10000 ×g for 10 min. The supernatant was collected and concentrated with Amicon (Millipore, USA) Ultra-4 centrifugal filter device. The concentrated samples were reconstituted to the original volume using the refolding buffer (50 mM Tris-Cl pH 8, 0.1 mM EDTA and 0.15 M NaCl) and centrifuged in a swinging bucket rotor (Kemi) at 3000-4000 ×*g* for approximately 10 min. The concentrated sample was given 5-6 washes with the refolding buffer to remove the denaturing salts and thus refolded the protein to its native form. The purified mf-PmCHH1 protein was measured using the Quant-iT™ protein assay kit using Qubit fluorometer (Invitrogen, UK)[17].

Western blot[19] was performed using anti-his antibody (Sigma, USA) to confirm the histidine tagged recombinant protein expressed. Briefly, the purified mf-PmCHH1 protein was transferred to nitrocellulose for Western blot. The nitrocellulose membrane was blocked with 5% skimmed milk in PBST, pH 7.2 [Phosphate buffered saline + Tween 20 (0.1 %)] for 1.5 h at room temperature. Incubated overnight at 4°C with primary antibody (Anti-His antibody-mouse) diluted (1:2000) (Sigma, USA) in 5% skimmed milk-PBST, followed by three washes with PBST (5-10 min each). The membrane was incubated in the secondary antibody, anti-goat IgG alkaline phosphatase conjugate (1:1000 dilution) (Sigma, USA) in 5% skimmed milk-PBST for 1-1.5 h with gentle agitation.The membrane was washed twice with PBST at a time interval of 5-10 min and detected by NBT/BCIP (Sigma) substrate. The colour development was observed and the membrane was washed in distilled water to stop the reaction. The colour development was visualized and documented using Gel documentation system (Gel Doc™ XR+ imaging system, Bio-Rad, USA).

Polyclonal antisera production, ELISA, Western blot and immunofluorescent detection

Eight-week old female balb/c mice (5-6) were immunized intraperitonialy with 100 µg of purified mf-PmCHH1 protein mixed 1:1 with complete Freunds adjuvant initially and subsequently with the same concentration of fusion protein in incomplete Freunds adjuvant. The boost injections were repeated

at a two week intervals and a test bleed was conducted prior to final bleeding[20]. The specificity and affinity of the polyclonal antisera produced was determined by Western blot analysis and immunofluorescence.

Polyclonal antisera against mf-PmCHH1 were analyzed for sensitivity by using enzyme-linked immunosorbent assay (ELISA). For anti-PmCHH1 polyclonal antisera concentration optimization assay by ELISA, the antigen mf-PmCHH1 (100 µL) was used to coat the 96-well micro titer plate and incubated overnight at 4°C. The free sites were blocked by coating the 96-well ELISA plate with 0.1 mL BSA (5% in PBS) for 1 h. Aliquots of different dilutions of antiserum (anti-mf-PmCHH1) starting from 1:200 to 1:409600 was added to each well following a double dilution series and incubated for 1 h. Triplicates were kept for each dilution. In the control wells, serum from the animal prior to immunization and PBS (Phosphate buffered saline) was added instead of antiserum. An aliquot of 0.1 mL of conjugated rabbit anti mouse IgG – HRP (1:2000 dilution) in 3% BSA/PBS was added and incubated for 1 h. TMB substrate (0.1 mL; 3,3',5,5'-tetramethyl-benzidine base, Sigma USA) was added and incubated for 10 min. The development of blue colour was observed and the reaction stopped by 50 µL of 1 M H_2SO_4. The yellow colour was measured at 450 nm using an ELISA reader (Tecan Infinite M200). The Abs_{450} measured was compared with the control serum. The plate was washed three times in between each step with PBST [phosphate buffered saline pH 7.2 with Tween 20 (0.01%)] [20].

ELISA was performed for standard curve and trend line analysis. Briefly, 100 µL of antigen (mf-PmCHH1) ranging from 0.00005 to 50 ng mL^{-1} were used to coat the 96-well micro titer plate and incubated overnight at 4°C. ELISA was performed as described earlier with a change in the antiserum concentration of 1:102400. The trend line obtained between the points were used for obtaining the equation of y = mx+C from which the value of x could be calculated to find the concentration of haemolymph CHH1 from unknown samples [20].

Western blot[19] was performed for the specificity of the polyclonal antisera raised in mice. The mature CHH1 fusion protein was run in 12% SDS-PAGE and the resolved protein was transferred to nitrocellulose membrane for Western blot. The nitrocellulose membrane was blocked with 5% skimmed milk in PBST, pH 7.2 (Phosphate Buffered Saline + Tween 20 (0.1 %)) for 1.5 h. Incubated overnight at 4°C with antisera raised against the mature Pm-CHH1 fusion protein (mf-PmCHH1), diluted (1:102400) in 5% skimmed milk-PBST, followed by three washes with PBST (5-10 min each). The membrane was incubated in the secondary antibody, antimouse IgG HRP conjugated (1:1000 dilution) (Sigma, USA) in 5% skimmed milk-PBST for 1-1.5 h with gentle agitation at room temperature. The membrane was washed twice with PBST at a time interval of 5-10 min. An aliquot of 4-chloronaphthol in 30 % H_2O_2 was added to the membrane and incubated for 10-30 min. The purple colour development was observed and the membrane was washed in distilled water to stop the reaction. The colour development was visualized and documented using Gel documentation system (Gel Doc™ XR+ imaging system, Bio-Rad, USA).

The eyestalks were cut from live anaesthetized shrimps (by dipping in ice flakes) and fixed in Davidson's fixative for 24 h for immunocytochemical analysis. The exoskeleton of the eyestalk was removed before dehydration and tissue embedded in paraffin using conventional methods. Consecutively, 5 µm sections were mounted onto the slide and dried at room temperature overnight. The tissue sections were dewaxed in xylene (twice for 10 min) and dehydrated through series of ethanol dilutions (70%, 95% and 100%). The sections were blocked with BSA (3% in PBS) for 1 h in a humified chamber. After blocking, the primary antiserum, anti-mf-PmCHH1 (1: 102400 dilutions in 3% BSA-PBS) was added and incubated overnight at 4°C. The secondary antibody, conjugate of anti mouse Ig G –FITC (Sigma, USA) (1:40 dilution) in 3% BSA prepared in PBS was added and incubated for 1 h in humified chamber (dark conditions) and finally stained with nuclear stain DAPI (10 µL, 0.02 µg mL^{-1}), incubated for 3 min. The slides were rinsed with distilled water, air dried and mounted with mounting media (Vectashield, USA) and observed under UV fluorescence microscope (Olympus, Germany). DAPI and FITC were viewed under different filters with excitation wavelength 360-370 nm and 470-490 nm, respectively. Between each step, the slides were washed twice for 5 min with PBST (PBS + 0.01 % Tween 20). The images were processed and merged using the "Imagepro - express" software (Media Cybernetics Inc, MD, USA) [20].

In vivo bioassay of mf-PmCHH1 fusion protein in _P. monodon_

P. monodon (10-15 g) obtained from local hatchery were stocked and maintained in a laboratory recirculating aquaculture system (RAS) for shrimp integrated with nitrifying bioreactor in seawater having 15 g L^{-1} salinity. Water quality was maintained within a narrow range (pH 6.8-7.8; total ammonia <0.1; nitrite <1.0; total alkalinity ($CaCO_3$) 75-125; and total hardness >5000-6000 mg L^{-1}). The animals were fed with commercially available pelleted feed (Higashimaru, India) containing 40% protein, 3% fat, 12% fiber, 18% ash and 12% moisture. The shrimps were in the D0-D1 stage during the time of injection. For *in vivo* bioassay of recombinant CHH1protein, the animals were eyestalk ablated 24 h prior to the injection.

The shrimps were bilaterally eyestalk ablated with quadrator and held under heavy aeration to recover the stress and returned to the respective tanks. After bilateral eyestalk ablation, the animals were starved for 18 h. The purified recombinant protein was dissolved in PBS at a concentration of 0.5 μg μL^{-1}. An aliquot of 100 μL of the thioredoxin fused mf-PmCHH1 protein solution was injected into the arthrodial membrane of the second walking leg[11]. Prior to injection, 25 μL of haemolymph was removed for baseline glucose measurement. Simultaneously, eyestalk ablated shrimps were injected with eyestalk extracts. To prepare the extract, the ablated eyestalks (stored at —80 °C) were used. The rigid exoskeleton and retina were removed prior to homogenization in PBS. The supernatant was injected into the eyestalk ablated shrimps as positive control. To compare, four sets of control were also considered: control 1 (100 μL PBS injected), control 2 (Uninjected), control 3 (100 μL total protein of *E. coli* BL21 (DE3) pLysS without vector), control 4 [100 μL total protein of *E. coli* BL21 (DE3) pLysS with pET32a+ vector without insert (pET32a+)].The haemolymph glucose levels were measured with glucose oxidase kit (Biolab Diagnostics (I) Pvt. Ltd, India). An aliquot of 2 μL of haemolymph was combined with 198 μL of glucose buffer and incubated for 15 min at 37°C. The glucose level was determined by measuring the absorbance at 500 nm. The level of CHH1 hormone was analyzed with antiCHH1 antisera by ELISA.

In vivo bioassay of antagonist (antisera) of mature fusion PmCHH1 protein in _P. monodon_

The shrimps (n=20) were starved for 18 h before the injection of anti-mf-PmCHH1 antisera. Prior to injection 25 μL of haemolymph was removed. The serum from mice containing antisera against mf-PmCHH1 protein was diluted 1:500 in PBS. Control serum was also diluted in the same manner (1:500). An aliquot of 100 μL of the diluted antiserum (anti-mf-PmCHH1 plus control serum) was injected into the pericardial cavity of *P. monodon* weighing 10-15 g (n=20) [11]. The haemolymph glucose levels were measured with glucose oxidase kit (Biolab Diagnostics (I) Pvt. Ltd, India). The haemolymph was collected at 0.5, 1.0, 1.5 and 2 h post injection. The haemolymph samples were centrifuged at 10000×*g* for 5 min at 4°C. An aliquot of 5 μL of haemolymph was combined with 195 μL glucose buffer and incubated for 15 min at 37°C. The glucose level was determined by measuring the absorbance at 500 nm and deducing the concentration from a standard graph. Circulating haemolymph CHH1 levels were analyzed using enzyme-linked immunosorbent assay (ELISA) with the polyclonal antisera of CHH1 (anti-mf-PmCHH1).

Statistical analysis

The data obtained in the *in vivo* bioassay of mature fusion Pm-CHH1 (mf-PmCHH1) protein and *in vivo* bioassay of the antagonist (antisera) of mature fusion Pm-CHH1 protein (anti-mf-PmCHH1) in *P. monodon* were subjected to ANOVA and Post-Hoc analysis (Tukey HSD) to bring out the significant differences. The statistical analysis was performed using the SPSS 11.5 software package.

Results and Discussion

Expression of thioredoxin fused mf-PmCHH1 protein, polyclonal antisera generation and immunolocalization of CHH1 neurosecretory cells

The presence of small quantity of CHH family neuropeptides in the eyestalk of shrimp poses a challenge for isolation of these peptides. Another factor is the similarity in the size and structure of the three CHH family neuropeptides which limit their isolation and purification directly from the animal[21]. The CHH1 expression translation vector construct could be built successfully for production of thioredoxin-fused mature CHH1 protein (mf-PmCHH1-29.47 kDa) in the *E. coli* BL21 (DE3) pLysS system by IPTG induction as an inclusion body. Using the lysis 2 extraction buffer, the protein expressed as an inclusion body was extracted (Fig. 1). Western blot of the expressed fusion protein with his-tag antibody demonstrated the estimated molecular

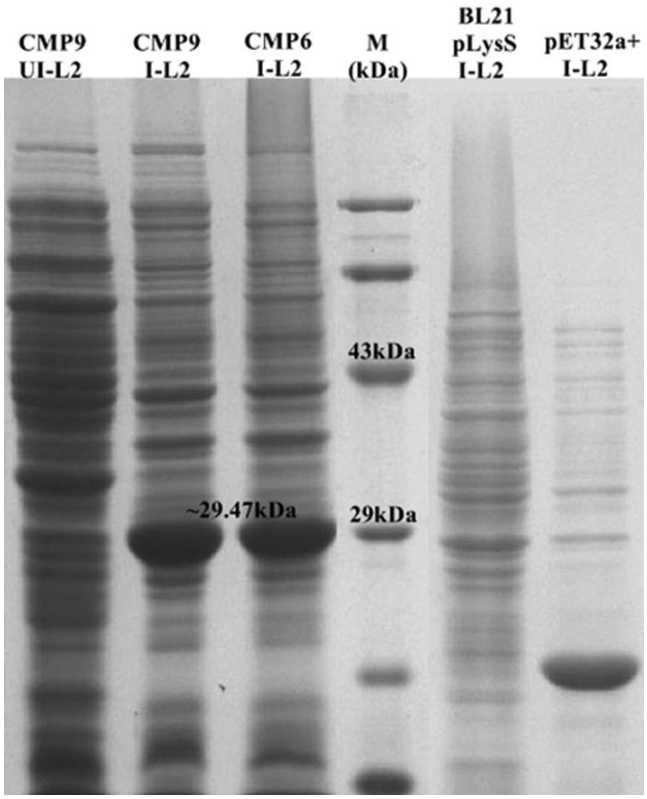

Fig. 1—Mature fusion PmCHH1 protein expressed in the induced culture is compared with uninduced culture, induced *E. coli* BL21 (DE3) pLysS culture with no vector and *E. coli* BL21 (DE3) pLysS transformed with pET32a+ without insert. [The proteins from all the samples were extracted with lysis buffer 2. The legends used - CMP9 (UI-L2) – Uninduced culture lysis 2; CMP9 (I-L2) - Induced culture lysis 2; CMP6 (I-L2) –Induced culture lysis 2; M (kDa)-Protein marker medium range; BL21-pLysS (I-L2) –Induced lysis 2; pET32a+ (I-L2) - Induced lysis 2]

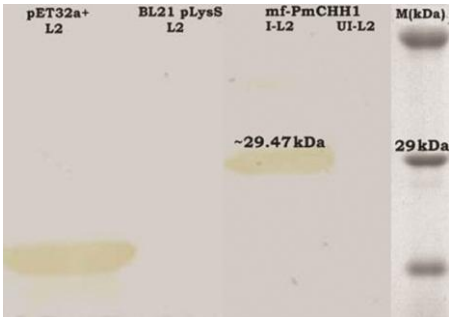

Fig. 2 — Western blot analysis with his-tag antibody of the induced mf-PmCHH1 compared with uninduced culture of mf-PmCHH1, induced *E. coli* BL21 (DE3) pLysS transformed with pET32a+ and *E. coli* BL21 (DE3) pLysS culture without vector. [The proteins from all the samples were extracted with lysis buffer 2. The legends used are pET32a+ L2; BL21 pLysS L2; mf-PmCHH1 (I-L2); mf-PmCHH1 (UI-L2); M (kDa)-Protein marker medium range. L2- designated for Lysis buffer 2, I- Induced, UI-Uninduced]

weight (29.47 kDa) of the mf-PmCHH1 protein (Fig. 2). The expressed fusion protein was further

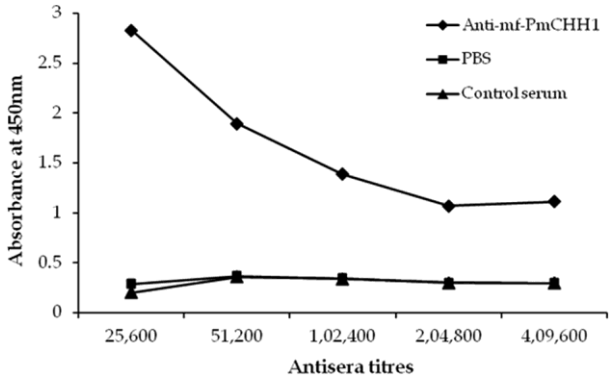

Fig. 3— Anti-mf-PmCHH1 polyclonal antisera concentration optimization assay by ELISA.

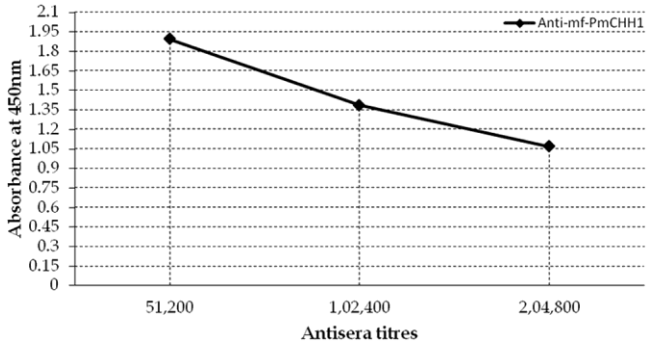

Fig. 4 — Linear response analysis for optimization of antisera titre (anti-mf-PmCHH1).

subjected to polyacrylamide gel extraction and refolding. The yield of PAGE purified and refolded mf-PmCHH1 protein was 4.31 mg mL^{-1} from 6 gel extracts. The yield of fusion protein (4.31 mg mL^{-1}) obtained is more in comparison to the *Pichia pastoris* expressed and purified Pem-CMG (260 µg L^{-1}) protein[10]. The purified and refolded fusion protein was used as antigen for raising polyclonal antisera in mice as well as for *in vivo* application studies in *P. monodon*.

The sensitivity of the polyclonal antisera was attained using ELISA; the absorbance value that reciprocates 1.0-2.0 units of OD was selected based on the anti-mf-PmCHH1 polyclonal antiserum concentration optimization assay (Fig. 3). The optimization assay specified that the titers ranging from 1:51200-1:204800 yield absorbance between 1.89-1.07 OD. A linear response was obtained between the 1.89 and 1.07 O.D (Fig. 4). Based on this optimization, the antisera titre of 1:102400 which yielded an absorbance of 1.39 OD was chosen for all the routine assays. The maximum titre of the antisera obtained was 1:409600. The antisera could

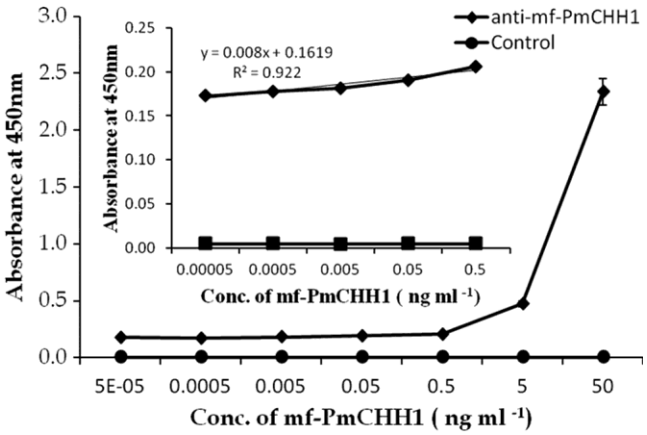

Fig. 5 — ELISA performed using polyclonal antisera anti-mf-PmCHH1 (1: 1, 02,400 dilution) and antimouse IgG HRP conjugate (1:1,000 dilution) (Sigma, USA) to confirm the sensitivity of the antisera raised against mf-PmCHH1 fusion protein expressed at concentrations ranging from 5×10^{-5} -50 ng mL^{-1} in comparison with the control serum. Inset graph is the trend line obtained between the points, displayed the equation of y = 0.008x+0.1619, R^2 = 0.922.

detect $5x10^{-5}$ – 50 ng ml^{-1} of purified mf-PmCHH1 at a dilution of 1:102400 (Fig. 5), the results obtained are comparable to anti-rPem-CHH1 antibody that could detect five nanograms of purified Pem-CHH1 at a dilution of 1:20000[14]. Western blot using polyclonal antisera of mf-PmCHH1 demonstrated the specific band from the total protein extracted of *E. coli* BL21 (DE3) pLysS bacterial cells transformed with mf-PmCHH1+pET32a+ expression vector and the PAGE purified, refolded mf-PmCHH1 protein (Fig. 6). This confirmed the identity of the fusion protein as mf-PmCHH1 protein. Further, dot-blot analysis also could detect 5×10^{-6} ng mL^{-1} of mf-PmCHH1 fusion protein (Fig. 7) revealing more specificity of the antisera generated against thioredoxin fused CHH1 protein (mf-PmCHH1). The simple technique of fusion of the target gene fragment to the 3' end of a translation vector to efficiently express the protein solves the difficulty in isolating the active neuropeptides from the animal[17, 21].

The immunofluorescence using the anti-mf-PmCHH1 antiserum demonstrated a scattered and mostly superficial distribution of CHH1 producing neurosecretory cells in the eyestalk of *P. monodon*. Distinct CHH1 producing neurosecretory cells were visible (Fig. 8A) in comparison with the control (Fig. 8B). The results that we attained with immunofluorescence agreed with the results obtained from *Cancer* crabs[22], *Carcinus maenas*[23], *Penaeus japonicus*[8], *Metapenaeus ensis*[21], *Nephrops*

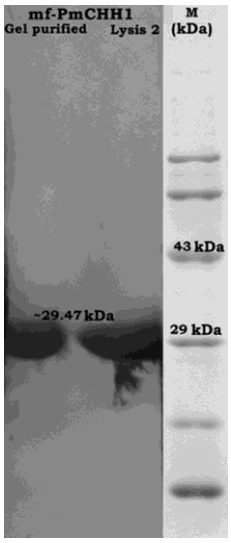

Fig. 6 — Western blot performed using polyclonal antisera anti-mf-PmCHH1 (1: 1, 02,400) and antimouse IgG HRP conjugate (1:1,000 dilution) (Sigma, USA) to confirm the specificity of mf-PmCHH1 fusion protein expressed. [The specificity was confirmed in PAGE purified, refolded mf-PmCHH1 protein (Lane 1) and total protein extracted from *E.coli* BL21 (DE3) pLysS bacterial cells transformed with mf-PmCHH1 expression vector (Lane 2)]

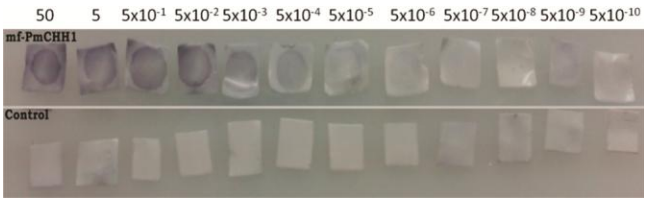

Fig. 7 — Dot-blot analysis performed using polyclonal antisera anti-mf-PmCHH1 (1: 1, 02,400 dilution) and antimouse IgG HRP conjugate (1:1,000 dilution) (Sigma, USA) to confirm the sensitivity of mf-PmCHH1 fusion protein expressed at concentrations ranging from 50-5×10^{-10} ng ml^{-1}in comparison with the control serum.

norvegicus[24], *Homarus gammarus*[7], and *Penaeus monodon*[17, 20]. The data revealed by these authors showed that neurosecretory cells producing CHH-family peptides were localized in the MTGX (medulla terminalis ganglion X organ). The anti-mf-PmCHH1 raised against the thioredoxin-fused mature CHH1 protein (mf-PmCHH1) can be used as a tool for immunolocalization of CHH1 neurosecretory cells in the eyestalk of *P. monodon.*

Biological functions of mf-PmCHH1 protein and polyclonal antisera (anti-mf-PmCHH1) in adult *P. monodon*

The thioredoxin-fused mature CHH1 protein (mf-PmCHH1) and the polyclonal antisera developed were tested in *P. monodon* over a short time period to determine their acute biological effects[14]. The biological activity of the mf-PmCHH1 protein was

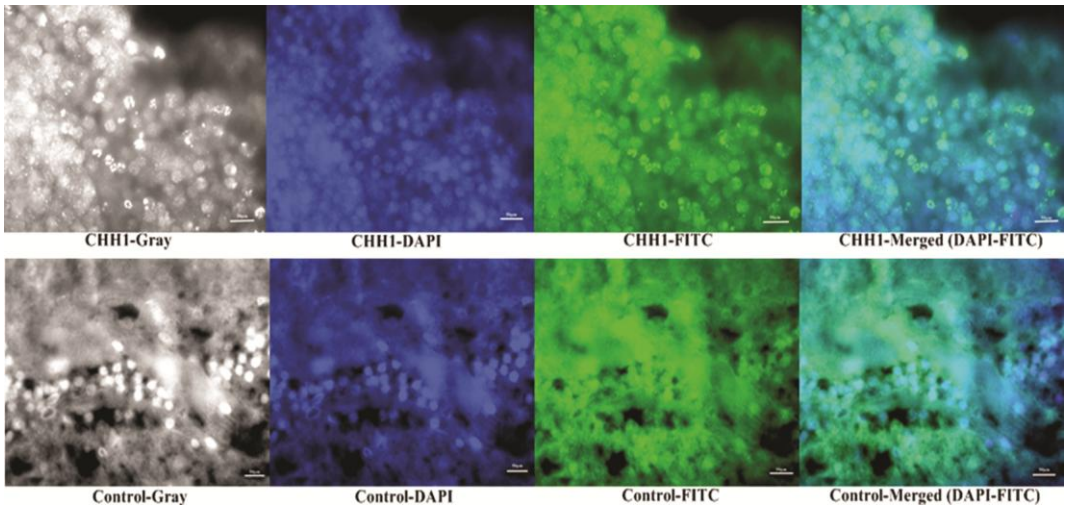

Fig. 8 — Immunolocalization of CHH1 neurosecretory cells in eyestalk using (A) antiserum-mf-PmCHH1 (1: 1, 02,400) and anti-mouse-FITC conjugate (1:40) in comparison with; and (B) control antiserum (mouse antiserum raised against PBS-1: 2000 dilution) and anti-mouse-FITC conjugate (1:40). [DAPI (nuclear stain), FITC (Fluorescein labelled secondary antibody)]

confirmed by the measurement of haemolymph glucose level. The hyperglycemic effect was observed within 0.5 h of injection that increased up to 80.59% (1.5 h) post administration in comparison with the three controls: control 1 (PBS injected), control 2 (uninjected) and control 3 (total protein of *E. coli* BL21 (DE3) pLysS without vector (BL21-pLysS), and also control 4 [total protein of *E. coli* BL21 (DE3) pLysS with pET32a+ vector without insert (pET32a+)] (Fig. 9). The increase in haemolymph glucose was similar to that of eyestalk extract injected positive control. The Post-Hoc analysis confirmed 1.5 h of administration to be a highly significant time period. The hyperglycemic levels produced are comparable to hyperglycemic activity obtained with recombinant Pem-CHH1, Pem-CHH2 and Pem-CHH3[10,11]. The Pem-CHH1, Pem-CHH2 and Pem-CHH3 recombinant protein were expressed in *Pichia pastoris*, while the fusion protein (29.47 kDa) of CHH1 is expressed in *E. coli* BL21 (DE3) pLysS. Besides being expressed in a prokaryotic system the thioredoxin fused CHH1 protein is able to show hyperglycemic activity through elevation in haemolymph glucose level of eyestalk ablated *P. monodon*. Morera *et al*.,[25] have shown that the fused (or chimeric) CHH protein of 63kDa expressed in *E. coli* elevated haemolymph glucose levels in eyestalk-ablated *Litopenaeus vannamei* (Boone) significantly 45 min post injection.

The fusion protein mf-PmCHH1 developed was used as the antigen for raising polyclonal antisera in mice. The antisera at a dilution of 1:500 were directly

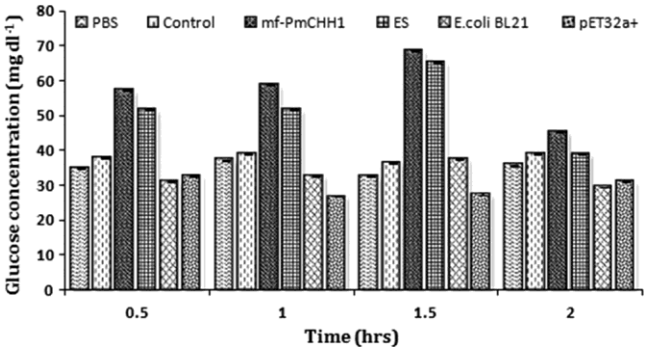

Fig. 9 — *In vivo* application of mf-PmCHH1 in *P. monodon* (n=20). [Observations of haemolymph glucose for a time period of 0.5, 1.0, 1.5 and 2.0 hours post application of mf-PmCHH1 fusion protein (5 μg)]

administered in *P. monodon* to assess the effect on regulation of glycemia. The *in vivo* application of mf-PmCHH1 antiserum (anti-mf-PmCHH1) at 1:500 dilutions resulted in a decreased hyperglycemic activity. Decreased haemolymph glucose and decreased haemolymph CHH1 levels were observed. A decrease in haemolymph glucose levels relative to the control was observed at 0.5 h (39%) and a decrease of 50% remained steady up to 2 h post injection (Fig. 6). In terms of haemolymph CHH1 level, 94.34% reduction was observed at 0.5 h and this remained almost steady (94.76%) for 2 h (Fig. 10). Therefore, the comparison between the haemolymph glucose and CHH1 levels of anti-mf-PmCHH1 and control at each time point evidenced 39-50% reduction in glucose concentration and 94.34-94.76% haemolymph CHH1 concentration. The results

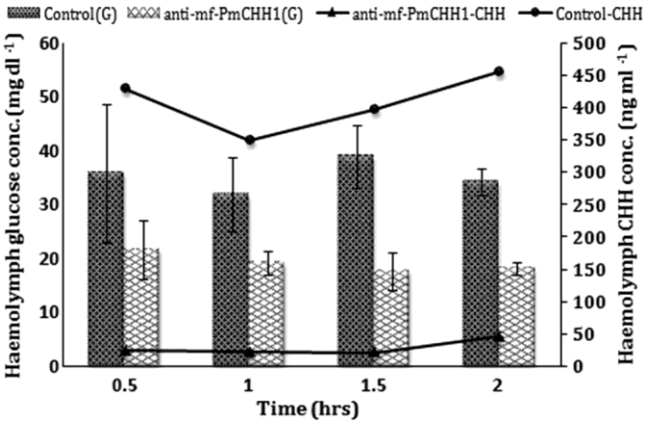

Fig. 10 — *In vivo* application of polyclonal antiserum of mf-PmCHH1 in *P. monodon* (n=20) at 1:500 dilution (27). [Observations of haemolymph glucose levels and CHH1 levels post application of antiserum of mf-PmCHH1 versus results from the control]

obtained are highly comparable to the anti-PemCHH1[14] that revealed about 30-50% reduction in the percentage of glucose concentration. This study did not reveal the concentration of haemolymph CHH1 on application of anti-PemCHH1. The Post-Hoc results also confirm the 1.5 h post administration of 1:500 dilutions of antisera to be a highly significant time interval in terms of haemolymph glucose and CHH1 levels. A process for generation of the thioredoxin-fused mature CHH1 protein (mf-PmCHH1) of *P. monodon* in a bacterial system, enabling production of large quantities of mf-PmCHH1 has been successfully developed, and the same has been used to raise polyclonal antisera (anti-mf-PmCHH1) having biological activity.

Conclusion

In conclusion, a thioredoxin-fused mature CHH1 protein (mf-PmCHH1) has been produced in *E. coli* and is useful as an antigen to generate polyclonal antisera. The two products developed are prominently effective tools for glycemic regulation during the maturation cycle of *Penaeus monodon* whilst variations in glucose level are of extreme importance. The biological activity expressed by the mature CHH1 protein (mf-PmCHH1) and polyclonal antisera (anti-mf-PmCHH1) demonstrates the post translational silencing effects that can be competently functionalized in achieving the goal of captive breeding in *P. monodon*. The post translation silencing effects can be visualized through immunolocalization of CHH receptors in the ovary, brain and nervous system enabling the investigation of CHH neurohormone signal transduction pathway for controlling and regulation of hyperglycemia. The antisera (anti-mf-PmCHH1) will be a resourceful tool for detection and profiling of crustacean hyperglycemic hormone levels in haemolymph at any given stage during the development of *P. monodon*.

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