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RESEARCH ARTICLE

Molecular cloning and expression of a putative crustacean hyperglycemic hormone of *Litopenaeus vannamei* in *Pichia pastoris*

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Keywords: CHH, *Litopenaeus vannamei*, *Pichia pastoris*, recombinant protein.

Abbreviations: CHH: crustacean hyperglycemic hormone
GIH: gonad-inhibiting hormone
MIH: molt inhibiting hormone
PBS: Phosphate Buffered Saline
PCR: Polymerase Chain Reaction
PVDF: Polyvinylidene Fluoride
TCA: Trichloroacetic Acid

Crustacean hyperglycemic hormone (CHH) is the most abundant and best studied member of the CHH/MIH/GIH neuropeptide hormone family. CHH plays a major role in controlling glucose levels in the hemolymph, and it also has significance in regulating molting, reproduction, and osmoregulation. In contrast, molt-inhibiting hormone (MIH) is responsible for maintaining animals in an intermolt stage. In this study, Liv-MIH-1 cDNA, which encodes a mature neuropeptide from the eyestalk of white shrimp, *Litopenaeus vannamei,* was expressed in methylotrophic yeast (*Pichia pastoris* KM71) under the control of an alcohol oxidase promoter. Recombinant Liv-MIH-1 was secreted into the culture medium using the α-factor prepro-sequence without Glu-Ala repeats. The expected protein, which had an apparent molecular mass of 12.1 kDa, was detected by Tricine-SDS-PAGE analysis and confirmed by Western blot. Pure recombinant Liv-MIH-1 was obtained by affinity chromatography, and N-terminal sequence analysis confirmed expression of the protein. Biological assays for CHH and MIH activity were also performed. Purified recombinant Liv-MIH-1 showed the ability to elevate the glucose level of hemolymph of *L. vannamei*, but molting was unaffected. Since these results are in agreement with the high structural and phylogenetic similarity that has been

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observed between Liv-MIH-1 and other CHH neuropeptides we propose to rename the protein Liv-CHH-SG1.

The X-organ sinus gland (XOSG) complex, located in the optic ganglia in the eyestalk, is the major neuroendocrine control center of crustaceans. Among the eyestalk peptides, crustacean hyperglycemic hormone (CHH) is the most abundant and the best studied. CHH is a member of a structurally related peptide family, which also includes molt-inhibiting hormone (MIH), gonad-inhibiting hormone (GIH) and mandibular organ-inhibiting hormone (MOIH) (Böcking et al. 2001). This peptide family plays important roles in controlling several physiologic processes such as regulation of growth and reproduction. CHH is involved mainly in the regulation of glucose levels in the hemolymph, as well as in the metabolism of carbohydrates and lipids, molt, reproduction and osmoregulation (Yasuda et al. 1994; Santos et al. 1997; Khayat et al. 1998; Serrano et al. 2003), while MIH is one of the major keys in mediating growth and reproduction (Webster and Chung, 1999; Sonobe et al. 2001). Neuropeptide members of the CHH family are difficult to study because they are synthesized in small quantities and the similarities in size and primary structure to other eyestalk neuropeptides limit their isolation and purification. DNA recombinant technology is useful in generating these crustacean peptides in sufficient quantities to enable further study of their physiological roles and structure-activity relationships.

CHH and MIH recombinant proteins have been successfully expressed in *Escherichia coli,* but they were aggregated in an insoluble form (Ohira et al. 1999; Gu et al. 2000; Gu et al. 2001; Gu et al. 2002). However, a Eukaryotic expression system offers advantages since solubilization and refolding are not necessary for the production of biologically active neurohormones. The methylotrophic yeast, *Pichia pastoris,* is attractive for expressing shrimp proteins due to its relatively simple expression methods for the secretion of recombinant proteins (Sun, 1997; Udomkit et al. 2004; Yodmuang et al. 2004). This methylotrophic yeast system can secrete high levels of a wide variety of heterologous proteins using the *Sacharomyces cerevisiae* α-factor prepro-sequence (Cereghino and Cregg, 2000). Additionally, Glu-Ala repeats enhance the activity of the Kex2 protease when the α-factor secretion signal is used (Raemaekers et al. 1999).

Five different cDNA sequences encoding MIH (Sun, 1994; Chen et al. 2007; Lago-Lestón et al. 2007) and one cDNA sequence encoding CHH (Lago-Lestón et al. 2007) have been reported in *Litopenaeus vannamei,* one of the most important cultured shrimp species in Latin America. The cDNA encoding Liv-MIH-1 has been cloned and sequenced recently by our group (Lago-Lestón et al. 2007). The gene was amplified using primers designed from the *mih* cDNA sequence reported by Sun (1994). However, structural and phylogenetic analysis suggested that Liv-MIH-1 may have CHH activity or have a multi-functional role as observed in other CHH family members (Chang et al. 1990; Sefiani et al. 1996; Lago-Lestón et al. 2007). Liv-MIH-1 was previously produced as a mature peptide in *Pichia pastoris* (data not published). However, the Glu-Ala repeats at the C-terminus of the α-factor prepro-sequence were not efficiently removed, resulting in polypeptides with heterogenous N-termini still containing N-terminal extensions. Incorrect processing of Glu-Ala repeats by the Ste13 aminopeptidase has been observed in other studies (Briand et al. 1999; Raemaekers et al. 1999). The present study, therefore, involves the construction and expression of a plasmid that contains the cDNA encoding Liv-MIH-1 peptide in *P. pastoris* using the α-factor prepro-sequence without Glu-Ala repeats. Additionally, the *c-myc* epitope, 6XHis tag, and enterokinase recognition sites were included at the C-terminus, for antibody detection, purification, and the possibility of subsequent elimination of the extra amino acids from the C-terminus of the recombinant Liv-MIH-1, respectively. SDS-PAGE, Western blot, and N-terminal peptide sequencing analyses confirmed the successful expression and N-terminal processing of the secreted protein. The biological assays for CHH and MIH activities of the recombinant Liv-MIH-1 are also described in this report.

MATERIALS AND METHODS

Materials

E. coli TOP 10F' and *P. pastoris* KM71 strains (Invitrogen, Carlsbad, CA, USA) were used for plasmid construction and expression, respectively. Recombinant Taq DNA polymerase, Zeocin, pPICZαA expression vector, EnterokinaseMax (EKMax) and the ProBond Purification system were purchased from Invitrogen; T4 DNA ligase was from Roche (Indianapolis, IN, USA), and media components were from BD (Franklin Lakes, NJ, USA). Broad Range Protein Molecular Weight Marker was from Promega (Madison, WI, USA).

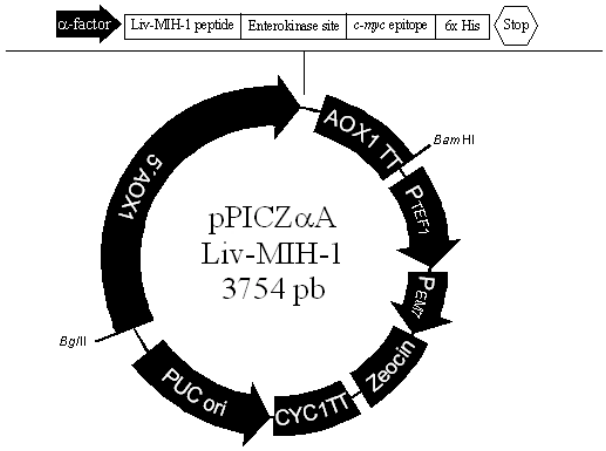

Figure 1. Map of pPICZαA-Liv-MIH-1.

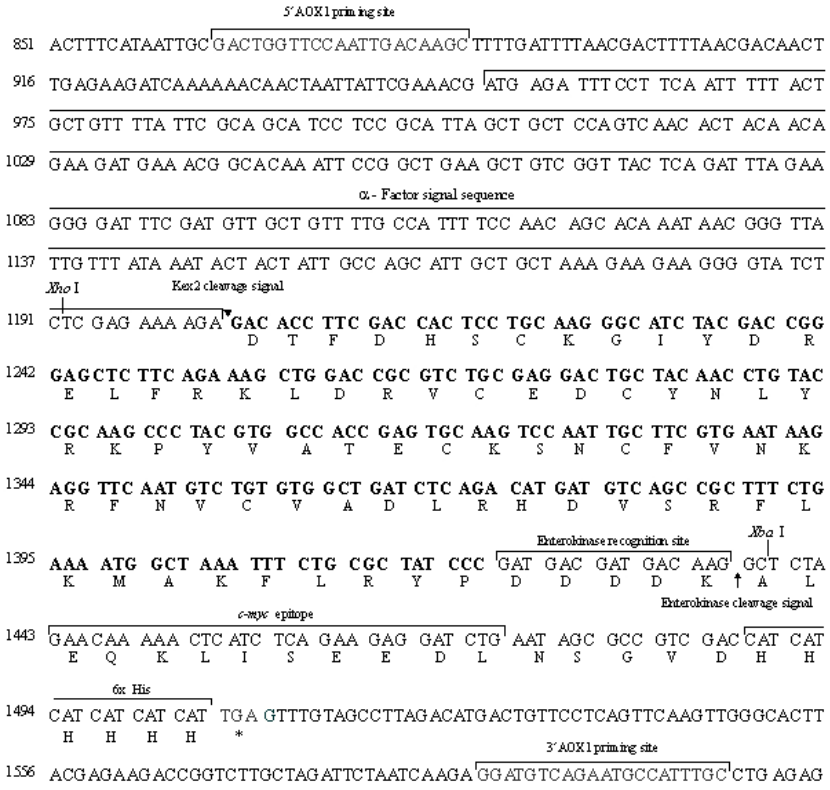

Figure 2. Nucleotide and deduced amino acid sequence of Liv-MIH-1. The mature Liv-MIH-1 peptide is bolded. The asterisk marks the termination codon.

Construction of the expression vector

The cDNA sequence encoding to the mature MIH peptide was amplified by PCR using primers that included the *Xho*I and *Xba*I sites and the enterokinase recognition site, the *c-myc* epitope, and the 6XHis tag. The primers were: F1: 5'CCGCTCGAGAAAAGAGACACCTTCGACCACTCC TGCAAGG3', R1: 5'GCTCTAGAGCCTTGTCATCGTCATCGGGATAGCG CAGAAA3'. The template was the recombinant plasmid clone pBAD-MIH. The PCR conditions were: initial denaturing at 94ºC for 3 min; 30 cycles of 45 sec at 94ºC, 30 sec at 55ºC, 30 sec at 72ºC; final extension at 72ºC for 10 min.

The amplified fragment of Liv-MIH-1 was digested with *Xho*I and *Xba*I and ligated into the corresponding restriction sites in the pPICZαA vector according to the manufacturer's protocol (Figure 1). Competent *E. coli* cells were transformed with the ligation product (pPICZαA-Liv-MIH-1) by electroporation, and transformants were selected in low salt Luria Bertani plates that contained 25 µg/ml Zeocin. The plasmid vector construction was verified by sequencing (SeqxCel, San Diego, CA, USA) using 5'AOX1 and 3'AOX1 primers (Invitrogen). The pPICZαA-Liv-MIH-1 vector was linearized with *BstX*I and introduced into *P. pastoris* KM71 by electroporation, as described in the manual (Version G) of the EasySelect *Pichia* Expression Kit (Invitrogen). The transformants were selected on YPDS plates (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar) that contained 100 µg/ml Zeocin and verified by colony PCR and sequencing analysis.

Expression of recombinants pPICZαA-Liv-MIH-1

A fresh, single colony of *P. pastoris* carrying pPICZαA-Liv-MIH-1 was grown in 3 ml of YPD medium (1% yeast extract, 2% peptone, 2% dextrose) with 100 µg/ml Zeocin and incubated for 24 hrs at 30ºC and 300 rpm. The culture was transferred into 100 ml of BMGY medium (1% yeast extract, 2% peptone, 0.67% Yeast Nitrogen Base (YNB), 4 µg/ml D-biotin, 100 mM potassium buffer pH 6.0, 1% glycerol) and incubated under the same conditions. When the OD_{600} of the culture reached 5, the cell culture was concentrated 5 times by centrifugation (2000 x *g*, 5 min). To determine the optimal conditions for Liv-MIH-1 expression, the *P. pastoris* pellet was resuspended in 20 ml of BMMY medium (1% yeast extract, 2% peptone, 0.67% YNB, 4 µg/ml D-biotin, 100 mM potassium buffer pH 6.0) with various concentrations of methanol (0, 1, 2 and 4%)

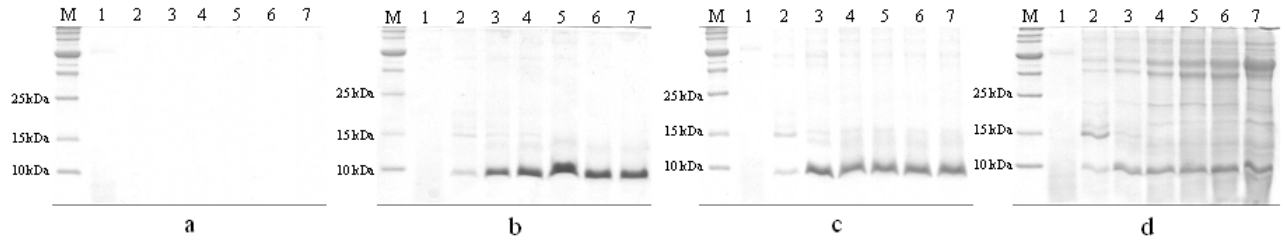

Figure 3. **Analysis of recombinant Liv-MIH-1 protein expression in *P. pastoris* by Coomassie-stained-Tricine-SDS-PAGE.** M: Broad Range Protein Molecular Weight Marker (Promega). Gels represent expression in different concentrations of methanol: a) 0%; b) 1%; c) 2% and d) 4%. Lanes 1 to 7 correspond to day of induction (0-6).

and monitored for 6 days. Fresh methanol was added daily to maintain induction. Every day, 1 ml aliquots of the expression cultures were collected; the samples were centrifuged (2000 x *g*, 5 min, 4ºC), and the supernatants were recovered and stored at -20ºC for further analysis.

Tricine–SDS-PAGE and Western blot analysis

The expression of the recombinant Liv-MIH-1 was analyzed by Tricine–SDS-PAGE (16.5%) according to the method of Schägger (2006). The secreted product was concentrated by TCA precipitation, resuspended in 20 µl of sample buffer, and heated in boiling water for 10 min before loading onto gel. The bands were visualized by staining with Coomassie Brilliant Blue R-250.

The presence of the recombinant Liv-MIH-1 was confirmed by Western blot analysis. The protein samples in the gel were transferred to a 0.45 µm Trans-Blot Transfer Medium nitrocellulose membrane (BioRad, Hercules, CA, USA) using the semidry blotting technique (Sambrook and Russell, 2001). Immunodetection was performed with an anti-*myc*-HRP antibody (Invitrogen). The membrane was soaked for 3 hrs at room temperature in PBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.05% (v/v) Tween-20, pH 7.4) with 5% (w/v) nonfat milk powder (Svelty), and continued for 2 hrs at room temperature in PBST containing 1% (w/v) nonfat milk powder and the anti-*myc*-HRP antibody (1:3500 dilution). The membrane was washed four times for 15 min with PBST. The signal was detected using Enhanced Chemiluminescence (ECL) reagents (Amersham Biosciences, Piscataway, NJ, USA).

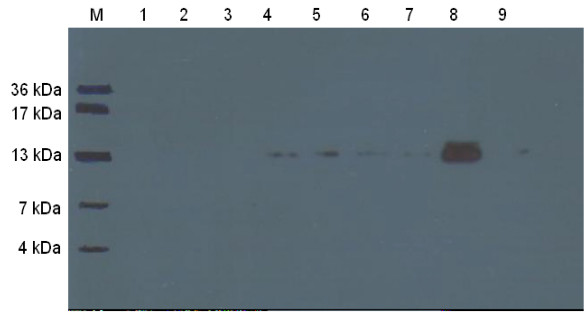

Figure 4. **Analysis of recombinant Liv-MIH-1 protein expression in *P. pastoris* by Western blot.** M: MultiMark Multicolored Standard (Invitrogen). Lanes 1 and 2 correspond to cultures induced with 2% MeOH: Lane 1, KM71; Lane 2, KM71 + pPICZαA without insert. Lane 3 corresponds to non-induced Liv-MIH-1. Lanes 4 and 5 correspond to Liv-MIH-1 induced with 0.5% MeOH: Lane 4, day 1; Lane 5, day 2. Lanes 6 and 7 correspond to Liv-MIH-1 induced with 2% MeOH: Lane 6, day 1; Lane 7, day 2. Lanes 8 and 9 correspond to Liv-MIH-1 induced with 4% MeOH: Lane 8, day 1; Lane 9, day 2.

Affinity-purification and N-terminal sequencing

The recombinant Liv-MIH-1 was purified by a Nickel-charged affinity column under native conditions according to the protocol of the ProBond Purification System (Invitrogen). Briefly, the supernatant from 50 ml of induced cell culture was adjusted to pH 8.0 and cleared by centrifugation at 3000 x *g* for 10 min. The supernatant was loaded onto a column (10 ml) containing 1 ml of ProBond Nickel-Chelating resin pre-equilibrated with the binding buffer (50 mM NaH_2PO_4, 500 mM NaCl, pH 8.0). The protein-bound resin was serially washed six times with native washing buffer (20 mM Imidazole, 50 mM NaH_2PO_4, 500 mM NaCl, pH 8.0). The protein was serially eluted 3 times in elution buffer (250 mM Imidazole, 50 mM NaH_2PO_4, 500 mM NaCl, pH 8.0). Fractions were analyzed by Tricine-PAGE and their concentration was determined with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The remainder of the eluted protein was concentrated using a 10 kDa cut-off concentrator (Orbital Biosciences, Topsfiel, MA, USA) and stored at -80ºC until use.

N-terminal sequencing was performed using affinity purified proteins blotted onto 0.45 µm BioTrace PVDF membrane (Pall, Ann Arbor, MI, USA) after separation by Tricine-SDS-PAGE as previously described. The protein bands were excised from the membrane and analyzed using

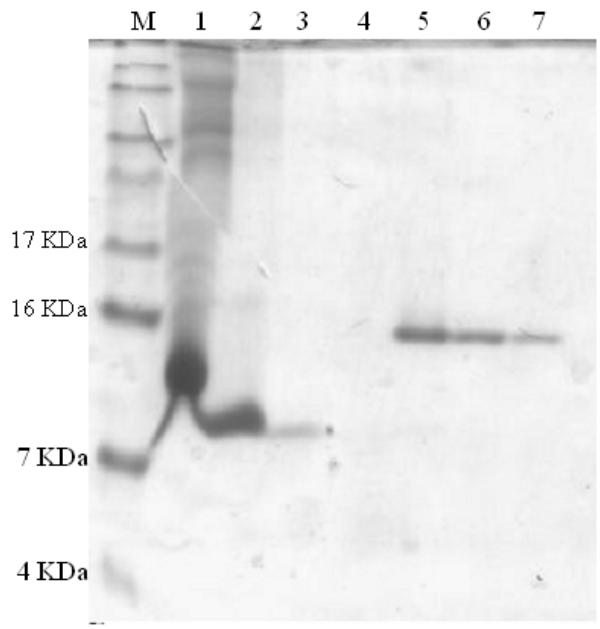

Figure 5. Analysis of affinity purified recombinant Liv-MIH-1 protein by Coomassie-stained-Tricine-SDS-PAGE. M: SeeBlue Pre-Stained Standard (Invitrogen). Lane 1 corresponds to the non-purified protein; Lanes 2 to 4 correspond to the samples obtained from the first three serial washes of the protein-bound resin; Lanes 5 to 7 correspond to the three serial elutions of Liv-MIH-1.

Edman degradation (Procise 491 Protein Sequencing System; Applied Biosystems, Foster City, CA, USA).

C-terminal removal

Purified recombinant Liv-MIH-1 (20 µg) was incubated overnight at 4ºC with 0.1, 0.5 and 1.0 U of EnterokinaseMax (Invitrogen), as specified by the manufacturer, to remove the *c-myc* epitope and the 6xHis tag at the C-terminus. A portion of the recombinant protein was similarly incubated with EnterokinaseMax buffer alone as a control. The enterokinase-treated Liv-MIH-1 recombinant protein was analyzed by Tricine–SDS-PAGE (16.5%) followed by staining with Coomassie Brilliant Blue R-250.

Bioassay for hyperglycemic activity

Juvenile *L. vannamei* white shrimp (18.4 ± 6.1 g) were maintained in individual containers (5 L) that were submerged in tanks with running seawater at 26ºC and 34‰ salinity. The animals were fed daily with a commercial diet. Individual shrimp were allowed to molt once before they were unilaterally ablated by cutting and cauterizing the eyestalk on the third day after molting. Individual shrimp that were starved for 12 hrs were injected with 2 µg of recombinant Liv-MIH-1 dissolved in 100 µl of PBS through the arthropodal membrane of the fifth walking leg by a syringe. For negative and positive controls groups, 100 µl of PBS and 100 µl of 1 eyestalk´s neuron equivalent crude extract on PBS was injected, respectively.

For glucose measurements, hemolymph (100 µl) was removed from the pleopod base of the first abdominal segment just prior to injection (baseline) and 1 hr after injection using a syringe with 200 µl of anticoagulant solution (10 mM EDTA, 450 mM NaCl, 10 mM KCl, 10 mM HEPES, pH 7.3) (Vargas-Albores et al. 1993). Hemolymph glucose concentrations were determined using a glucose oxidase diagnostic kit (Eagle Diagnostic, De Soto, TX, USA). Hyperglycemic activity was assessed by measuring the increase in hemolymph glucose levels following injection. The data were tested for homogeneity of variances and normality (Zar, 1999). Statistical analysis of the data was performed using one-way ANOVA.

Bioassay for molt-inhibiting activity

Thirty juvenile shrimp (16.2 ± 1.3 g) were divided into three groups of 10 organisms each, and they were maintained using the same culture conditions as described for the hyperglycemic assay. On the third day after the first molt, individual shrimp were injected with 2 µg of recombinant Liv-MIH-1 dissolved in 100 µl of PBS through the arthropodal membrane of the fifth walking leg using a syringe. The shrimp in the control group were injected with 100 µl of PBS. A second control group comprising uninjected shrimp was included. Shrimp were inspected daily for mortality and molting. The duration of the molt cycle was recorded. Statistical analysis of the data was performed using one-way ANOVA.

RESULTS AND DISCUSSION

Construction of the *P. pastoris* expression vector containing Liv-MIH-1

For the construction of the pPICZαA-Liv-MIH-1 recombinant plasmid, Liv-MIH-1 cDNA from the pBAD-MIH expression vector was subcloned into the pPICZαA vector using R2 and F2 primers (Figure 1). No mutations were found in the nucleotide sequence of the inserted fragment. The mature MIH peptide sequence was inserted in frame with the α-factor protein secretion signal and the C-terminal recognition sites. The DNA sequence of the pPICZαA-Liv-MIH-1 vector predicts that the recombinant protein will contain 101 amino acids: 73 amino acids in the mature MIH peptide and the remaining 28 amino acids comprising the enterokinase recognition site, *c-myc* epitope, and 6XHis tag (Figure 2). The expected molecular weight of the recombinant product is 12.1 kDa.

Approximately 30 transformants of the KM71 strain were generated. Twenty clones were isolated and ten of them were screened by PCR. All of the clones contained the expected 750 bp DNA fragment, indicating that the Liv-MIH-1 gene was integrated into the *P. pastoris* genome.

Table 1. Molt cycle duration in the white shrimp *L. vannamei* after injection of recombinant Liv-MIH-1, PBS and uninjected. Duration was expressed as days ± SD. Statistical analysis was performed by one-way ANOVA (p = 0.7).

Group	Number (n)	Duration (days)
Liv-MIH-1 (2 µg)	10	14.8 ± 1.3
PBS	10	14.8 ± 1.3
Uninjected	10	15.2 ± 1.2

Liv-MIH-1 expression

The recombinant Liv-MIH-1 was expressed and secreted into the culture medium. The peptide was analyzed by Tricine-SDS-PAGE and the band corresponding to the expected size (12.1 kDa) was visible on the gel. This protein band was not detected in non-induced cultures (0% MeOH) or in the culture of the transformant harboring pPICZαA only. The expression of the recombinant Liv-MIH-1 protein correlated with the induction time and MeOH concentration; induction with 4% MeOH for 1 day was optimal. No remarkable differences were observed with a greater induction time (Figure 3). Western blot analysis using the anti-*myc*-HRP antibody confirmed that the band observed corresponds to the expected recombinant protein (Figure 4).

Recombinant protein purification and sequencing

All contaminating proteins were removed by affinity purification of Liv-MIH-1 (Figure 5). The final yield of the recombinant Liv-MIH-1, after purification, was 8.7 mg/l of culture medium. N-terminal sequencing revealed that the first five amino acid residues of the secreted recombinant protein were DTFDH, which were identical to the mature Liv-MIH-1 protein. This confirmed that the α-factor prepro-sequence of Liv-MIH-1 was successfully cleaved by the Kex2 protease without Glu-Ala repeats. Although the presence of Glu-Ala repeats enhances the activity of the Kex2 protease when the α-factor secretion signal is used, the subsequent processing of these repeats by the Ste13 aminopeptidase is inefficient in some cases, resulting in extra amino acid residues in the recombinant protein (Briand et al. 1999; Raemaekers et al. 1999). The correct processing of the α-factor without Glu-Ala repeats has been also shown in other previous studies (Briand et al. 1999; Raemaekers et al. 1999; Treerattrakool et al. 2002). The correct processing of the α-factor prepro-sequence at the N-terminus may be important for the correct folding and biological activity of the recombinant Liv-MIH-1 protein (Gong and Clark-Lewis, 1995). However, the presence of 20 additional amino acids in the N-terminus of the recombinant CHH from *Metapenaeus ensis* had no effect on the hyperglycemic activity (Gu et al. 2000).

C-terminal removal

Once the recombinant Liv-MIH-1 protein was purified by an affinity column using the 6xHis tag and the band was confirmed by the anti-*myc*-HRP antibody directed at the *c-myc* epitope, different concentrations of the enterokinase enzyme were used to remove the *c-myc* epitope and the 6xHis tag from the C-terminus of Liv-MIH-1. As can be observed in Figure 6, partial digestion products were obtained with all the enterokinase treatments. However, the digestion clearly allowed to distinguish the 22 amino acids fragment that was removed from the C-terminus of the recombinant protein, leaving the 6 amino acids of the enterokinase recognition site (Figure 2). This suggests a high specific cleavage site recognized by enterokinase. Enterokinase cleavage could be useful for recombinant proteins in which the His tag and the *c-myc* epitope of the C-terminus interfere with the biological activity of the proteins.

Biological assay of recombinant Liv-MIH-1

The hyperglycemic effect of injection with PBS, eyestalk extract, and recombinant Liv-MIH-1 on the hemolymph of unilaterally ablated *L. vannamei* was recorded 1 hr after injection (Figure 7). In shrimp that were injected with recombinant Liv-MIH-1, an increase in hemolymph glucose level was detected (12.9 $mg \cdot dl^{-1}$). This level was lower than that of shrimp that were injected with eyestalk

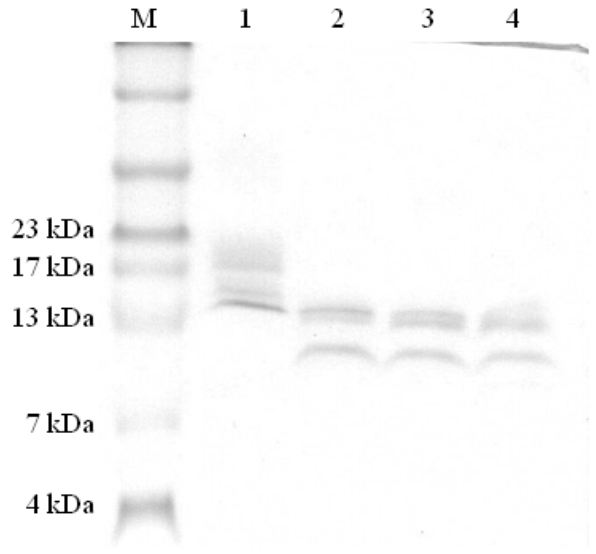

Figure 6. Analysis of enterokinase-treated Liv-MIH-1 recombinant protein by Coomassie-stained-Tricine-SDS-PAGE. M: MultiMark Multicolored Standard (Invitrogen). Lane 1 corresponds to the undigested purified protein; Lanes 2 to 4 correspond to the purified protein digested with enterokinase: Lane 2, 0.1 U; Lane 3, 0.5 U and Lane 4, 1 U.

extract (19.7 mg·dl^{-1}) but higher than PBS-injected shrimp (4.7 mg·dl^{-1}) ($p < 0.001$).

The ability of recombinant Liv-MIH-1 to elevate glucose levels in the hemolymph of *L. vannamei* clearly indicates that the recombinant Liv-MIH-1 has hyperglycemic activity. This model is consistent with the primary structure of Liv-MIH-1 and its precursor -*i.e.*, the lack of glycine at position 12 of the mature peptide compared with those of the MIH/GIH group; the presence of a CHH precursor–related peptide (CPRP) and the presence of the motif A1 (Lago-Lestón et al. 2007). All of these characteristics have been described for type I peptides of the CHH family (de Kleijn and van Herp, 1995; Lacombe et al. 1999; Böcking et al. 2001) and are in agreement with the high sequence similarity of other CHH neuropeptides that was documented in a phylogenetic analysis by Lago-Lestón et al. (2007). Interestingly, the analysis of the nucleotide sequence of Liv-MIH-1 and Liv-MIH-2 isoforms suggested that the *mih* gene from *L. vannamei* consist of 4 exons and 3 introns (Lago-Lestón et al. 2007). This exon structure is in agreement with the 4-exon organization observed in *chh* genes (type I) of a wide range of crustacean and hexapod. Meanwhile, the 3-exon structure has been found in *mih*/*gih* genes (type II) of crustacean species (Chen et al. 2005).

Furthermore, high gene expression levels of Liv-MIH-1 have been observed under extreme conditions of salinity (between 10‰ and 40‰) (Lago-Lestón et al. 2007). At this range of salinity, shrimp require active processes to compensate for changes in internal osmotic concentration (Díaz et al. 2001). In this regard, the hyperglycemic activity that was observed with Liv-MIH-1 concurs with the role of CHH in other processes such as osmoregulation (Serrano et al. 2003).

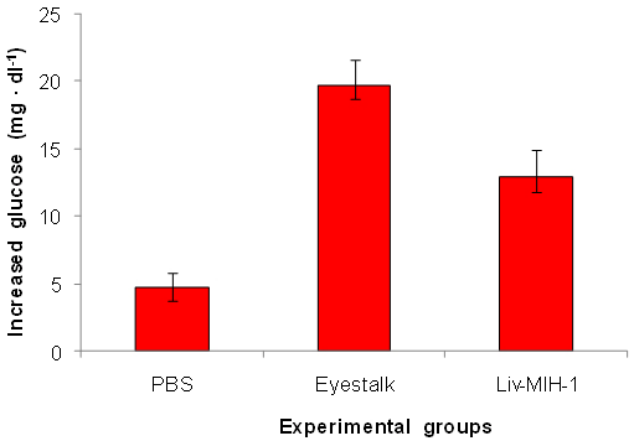

Figure 7. Increased glucose level in the hemolymph of unilaterally ablated *L. vannamei* at 1 hr after injection with PBS, crude extract from one eyestalk and 2 µg of recombinant Liv-MIH-1. Results are expressed as the mean ± SD. Statistical analysis was performed by one-way ANOVA ($p < 0.001$).

That the hyperglycemic effect of the recombinant protein was not comparable with that of the crude eyestalk extract can be explained by the presence of 22 additional amino acids in the C-terminus, corresponding to the enterokinase recognition site, the *c-myc* epitope and the 6xHis tag. Additionally, the lack of carboxy-terminal amidation can also affect the biological activity of recombinant proteins. However, recombinant *M. ensis* CHH molecules (rMeCHH-A and rMeCHH-B), which have 27 additional amino acid residues at the N-terminus and a free C-terminus, show hyperglycemic activity that is similar to that observed for crude eyestalk extracts (Gu et al. 2000). In contrast, low hyperglycemic activity was showed by recombinant *P. japonicus* CHH (rPej-SGP-I), which has 26 additional amino acid residues at the N-terminus and an amidated C-terminus (Katayama et al. 2002). The authors also suggested that in addition to the C-terminal amide moiety, the amino acid sequence at the C-terminus may be necessary for conferring hyperglycemic activity.

In our study, the moderate hyperglycemic activity that was elicited by recombinant MIH-Liv-1 may be ascribed to the extra amino acids at the C-terminus, which can partially affect MIH-Liv-1 activity. It is possible, then, that the CHH activity of Liv-MIH-1 would be improved after removing the His tag and *c-myc* epitope from the C-terminal region of the recombinant peptide through enzymatic cleavage by enterokinase.

The inhibiting effect of Liv-MIH-1 on molting was also observed *in vivo* in non-ablated *L. vannamei* shrimp. The molt cycle duration of shrimp that were injected with recombinant Liv-MIH-1 was similar to the control (PBS) and uninjected groups (Table 1); no statistical differences were detected by one-way ANOVA ($p = 0.7$). Because the molt cycle duration of Liv-MIH-1-injected shrimp was not longer than in control groups, recombinant Liv-MIH-1 may have not an inhibiting effect on molting.

In a similar bioassay using 1 µg of recombinant *M. ensis* MIH, which has extra amino acids at its N-terminus, the molt cycle duration was extended, and molt-inhibiting activity was proposed for this recombinant peptide (Gu et al. 2001). These results suggest that extra amino acids at the N-terminus are not critical for the MIH activity, but we can not discard the possibility that the extra amino acids at the C-terminus of Liv-MIH-1 could affect the conformation of the protein or the accessibility to the functional MIH site.

The structure of *Marsupenaeus japonicus* MIH consists of five α–helices and no β-structures (Katayama et al. 2003). The functional site of MIH is located in a region that contains the C-terminal sides of the α1 and α5 helices (Katayama et al. 2004), which suggests that the extra amino acids in Liv-MIH-1 could be affecting MIH activity. An assay that examines Liv-MIH-1 without its extra amino acids should clarify whether Liv-MIH-1 has or does not have molt-inhibiting activity. Because CHH activity triggers important events in physiological processes, such

as carbohydrate and lipid metabolism, reproduction, and osmoregulation, recombinant Liv-MIH-1 will facilitate studies on the physiological roles and structure-activity relationships in the eyestalk neurohormones of *L. vannamei*.

Previously we showed that sinus gland Liv-MIH-1 and Liv-MIH-2 are more related to the CHH group that to the MIH/GIH/MOIH group (Lago-Lestón et al. 2007). We now show that the recombinant Liv-MIH-1 had hyperglycemic activity so we propose to rename the protein Liv-CHH-SG1, and Liv-MIH-2 as Liv-CHH-SG2. This will avoid confusion with the Liv-MIH-1 and Liv-MIH1 peptides reported by Chen et al (2007), which were also obtained from the eyestalk ganglia of *L. vannamei,* but belong to the MIH/GIH/MOIH group and function as MIHs.

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