hyperglycemic hormone (CHH), molt-inhibiting hormone (MIH), and gonad-inhibiting hormone (GIH) (Charmantier et al., 1997), CHH has been widely studied as a result of its abundance and the well-established assays for biological activity (Leuven et al., 1982; Gu et al., 2000). Although CHH functions mainly in the regulation of glucose metabolism, recent studies have indicated that it might also play a role in reproduction and molting.

The primary structure of a hormone with both hyperglycemic activity and molt-inhibiting activity (CHH-A) has been described in *Homarus americanus* (Chang et al., 1990). Moreover, *H. americanus* CHH-A is also involved in triggering the onset of vitellogenesis, whereas CHH-B is responsible for stimulating oocyte maturation before spawning (De Kleijn et al., 1995, 1998). In *Metapenaeus ensis*, an important role of CHH-related neuropeptides, so-called MeCHH-A and MeCHH-B, in regulation of glucose metabolism and regulation of vitellogenesis in the female has also been demonstrated (Gu et al., 2000). A high level of MeCHH-A may be needed for the initial gonadal stage, and a high level of MeCHH-B is required for gonad maturation during middle and late vitellogenesis (Gu et al., 2000). In addition, CHH has been implicated in the regulation of lipid hepatopancreatic enzyme secretion and gill ion transport (Van Herp, 1998; Spanings-Pierrot et al., 2000).

In *Penaeus monodon*, 6 complementary DNA sequences encoding peptides in the CHH/MIH/GIH family have been reported so far (Davey et al., 2000; Udomkit et al., 2000). The biological activity of the peptides translated from these cDNAs has yet to be demonstrated. Though several recombinant peptides in this family, such as CHH and MIH of *M. ensis* (Gu et al., 2000, 2001) and MIH of *P. japonicus* (Ohira et al., 1999), were successfully expressed in *Escherichia coli* expression system, the expressed proteins aggregated in an insoluble form. The problems of complicated downstream processes resulting from *E. coli* expression systems such as solubilization and renaturation of the recombinant protein could be overcome by using eukaryotic expression systems, among which *Pichia pastoris* is one of the most powerful for synthesizing heterologous proteins (Higgings and Cregg, 1998). *Pichia pastoris* is a methylotrophic yeast that can grow on methanol as a sole carbon source. The first step in the methanol utilization pathway is catalyzed by alcohol oxidase, which accounts for more than 30% of total cellular proteins. The highly inducible promoter of the alcohol oxidase I (*AOX1*) gene has been used for efficient production of a wide variety of heterologous proteins (Cereghino and Cregg, 2000).

In this study a recombinant peptide in the CHH/MIH/GIH family of *P. monodon*, so-called Pem-CMG, from *P. pastoris* in the form of secreted protein was expressed. The purification and biological assay for CHH activity of the recombinant Pem-CMG peptide are also described in this report.

Materials and Methods

Construction of an Expression Plasmid

A DNA fragment containing the coding sequence for the mature Pem-CMG was obtained by polymerase chain reaction (PCR) amplification from the plasmid Pem-CMG-EX2.1, which contains the mature Pem-CMG cDNA inserted in pET3a expression vector (Chooluck et al., 2000). The amplified DNA fragment was then cloned into the pPICZαA expression vector (Invitrogen).

The resulting recombinant plasmid, αCMG, contains the coding sequence for mature Pem-CMG, fused in-frame with the α-factor secretion signal of *S. cerevisiae* without the double Glu-Ala repeats, under the control of *AOX1* promoter. The physical map of the recombinant plasmid αCMG is shown in Figure 1.

Transformation of *Pichia pastoris* by Electroporation

The recombinant plasmid αCMG was linearized by *Pme*I, and used for transformation of *P. pastoris* strain KM71 (Invitrogen) by electroporation (Gene Pulser, Bio-Rad), with the following conditions: 1.5 kV, 25 μF, and 200 Ω. After the addition of 1 ml of 1 M sorbitol, the cells were incubated at 30°C without shaking for 1 hour. Then 1 ml of YEPD (1% yeast extract, 2% peptone, 2% glucose) was added, and the incubation was continued with shaking at 30°C for 2 hours. The cells were collected and resuspended in 100 μl of sterile distilled water. The cell suspension was spread on a YEPD plate containing 100 μg/ml Zeocin and incubated for 2 to 3 days until colonies formed. The transformants were screened for integration of αCMG into the genome by PCR amplification with 5′ and 3′ *AOX1* primers (5′ *AOX1*, 5′-GACTGGTTCCAATTGACAAGC-3′; 3′ *AOX1*, 5′-GCAAATGGCATTC TGACATCC-3′).

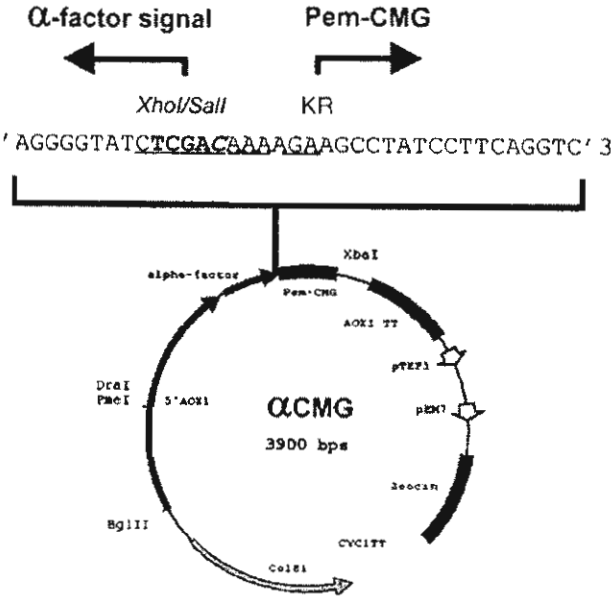

Figure 1. Physical map of αCMG. The recombinant αCMG plasmid contains the mature *Pem-CMG* cDNA fused in-frame with the α-factor secretion signal without Glu-Ala repeats. Expression of the mature Pem-CMG is driven by the AOX1 promoter in the 5′ AOX region. The expanded region spans the junction between the α-factor secretion signal and the mature Pem-CMG cDNA, in which the KEX2 cleavage site (KR) at the 3′ end of the the α-factor secretion signal is followed directly by the mature Pem-CMG cDNA.

Expression of Recombinant *Pem-CMG*

A single colony of *P. pastoris* KM71 recombinant containing αCMG was inoculated in 5 ml of YEPD and incubated at 30°C with shaking at 250 rpm for 48 hours, following which the cell culture was transferred into 100 ml of fresh BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 0.67% YNB, 4×10^{-5}% biotin, 1% glycerol) and grown under the same conditions until the culture reached an OD_{600} nm of 5 to 6. The cell pellet was harvested and resuspended in BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 0.67% YNB, 4×10^{-5}% biotin, 0.5% [vol/vol] methanol) using one-fifth volume of the original culture. Absolute methanol was added to a final concentration of 3% (vol/vol) to provide induction for 3 days. The expressed recombinant Pem-CMG secreted into the culture supernatant was analyzed on 16.5% Tricine gel for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the amino acid sequence at the amino terminus was determined by sequencing.

Purification of Recombinant *Pem-CMG*

After 3 days of induction, the culture supernatant was subjected to partial purification by precipitation with 30% to 50% ammonium sulfate and concentrated by ultrafiltration using Centriprep centrifugal filter devices (Amicon). Further purification was achieved by reverse-phase high-performance liquid chromatography (RP-HPLC) on Jupiter Phenomenex C18 column (Phenomenex, 5 μ particle, 300 Å pore size, 250 × 4.6 mm). The column was eluted with a linear gradient of 0% to 10% acetonitrile in 0.1% (vol/vol) trifluoroacetic acid (TFA) for 1 minute, then held at 10% acetonitrile in 0.1% (vol/vol) TFA for 10 minutes, followed by a linear gradient of 10% to 60% acetonitrile in 0.1% (vol/vol) TFA for 40 minutes, and finally held at 60% acetonitrile in 0.1% (vol/vol) TFA for 10 minutes at a flow rate of 1 ml/min. The expected fraction was collected and then freeze-dried. The purified protein was dissolved in phosphate-buffered saline (PBS), pH 7.4, and used in the biological activity assay.

Biological Assay for Hyperglycemic Activity of Recombinant *Pem-CMG*

Farm-grown black tiger prawns (*P. monodon*, 16–25 g) were kept in Plexi Glass tanks filled with seawater (approx 11 ppt of salinity) at a depth of 8 to 10 cm with good aeration for 3 to 4 hours. Prawns were then bilaterally eyestalk-ablated with a pair of sharp scissors, the wounds were immediately sealed with hot forceps, and the prawns were returned to the tanks.

After bilateral eyestalk ablation the prawns were starved for 18 hours, following which 100 μl of hemolymph was removed from individual prawns for the measurement of baseline glucose levels. For each experimental and control group, 10 prawns were used. Either 5 μg of purified recombinant Pem-CMG or 25 μg of total crude recombinant Pem-CMG protein in a volume of 100 μl was injected into an individual prawn through the arthrodial membrane of the second walking leg. A 100-μl volume of PBS and 1 pair-equivalent of eyestalk crude extract was injected into the negative and positive control groups, respectively. After injection the prawns were returned to the tanks, and the glucose levels in the hemolymph were determined at 0.5, 1, and 1.5 hours after injection using a glucose diagnostic kit (Sigma).

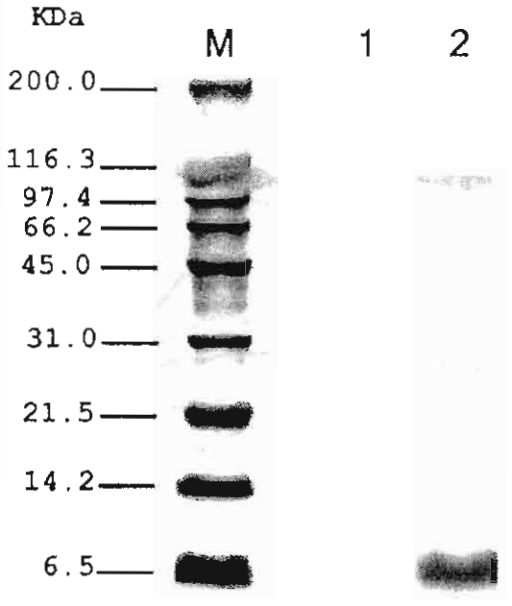

Figure 2. Coomassie blue–stained tricine SDS-PAGE analysis of the expression of the recombinant Pem-CMG protein from *P. pastoris* transformant. Lane M shows a broad-range protein marker (Bio-Rad). A 100-µl volume of the culture supernatant of the *P. pastoris* transformant containing pPICZαA vector only and αCMG after induction with 3% (vol/vol) methanol for 3 days were precipitated with TCA and loaded in lane 1 and lane 2, respectively.

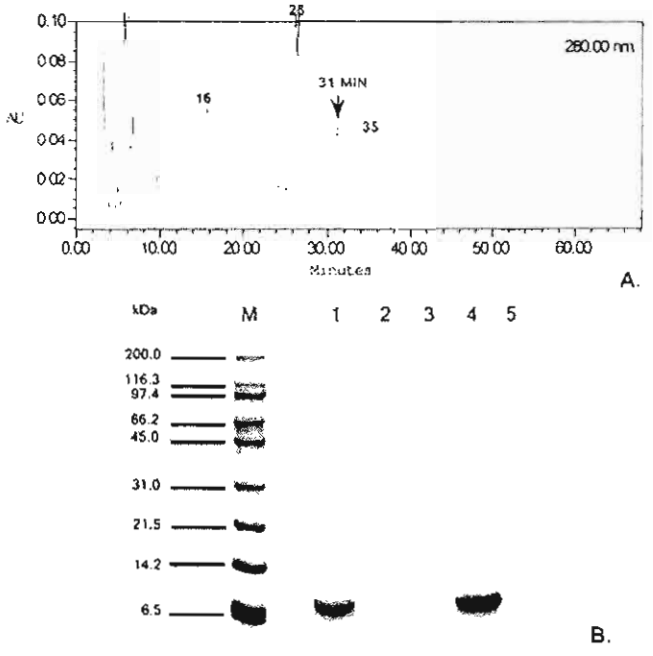

Figure 3. Purification of recombinant Pem-CMG. A: HPLC analysis of the partially purified recombinant Pem-CMG at wavelength of 280 nm. B: Tricine SDS-PAGE of 3 µg of the partially purified Pem-CMG from 30% to 50% ammonium sulfate precipitation (lane 1), and RP-HPLC purified fractions at retention times of 16, 26, 31, and 35 minutes (lanes 2, 3, 4, and 5, respectively). Lane M shows a broad-range protein marker (Bio-Rad).

Results

Analysis of *Pem-CMG* Expression in *Pichia pastoris*

An SDS-PAGE analysis of the culture supernatant of *P. pastoris* transformant containing αCMG showed a major protein product of about 8 kDa, which is the expected size for Pem-CMG (Figure 2, lane 2). This protein band was not present in the culture supernatant of *P. pastoris* transformant containing pPICZαA vector alone (Figure 2, lane 1). The result of amino-terminal sequencing of this protein showed that the first 5 amino acid residues were Ser-Leu-Ser-Phe-Arg, corresponding to the amino acid sequence deduced from cDNA encoding the mature Pem-CMG.

Purification of Recombinant *Pem-CMG*

Partial purification by ammonium sulfate precipitation was successful in removing some endogenous proteins from the culture supernatant of *P. pastoris* transformant containing αCMG, as shown in Figure 3 (B, lane 1). The RP-HPLC elution profile of the partially purified Pem-CMG and the result of Tricine SDS-PAGE (Figure 3 A and B) showed that a protein of the expected size for Pem-CMG eluted at 31 minutes (B, lane 4). The expected fraction was reloaded onto RP-HPLC column under the same conditions as the previous step so as to confirm the purity of the purified Pem-CMG protein. The chromatogram and Tricine SDS-PAGE revealed that Pem-CMG eluted as a single population at 31 minutes, and no contaminating proteins co-eluted at this stage (data not shown). The final yield of the purified Pem-CMG was 260 µg/L of the culture medium as determined by Bradford's method using the Bio-Rad Protein Assay kit.

Hyperglycemic Activity of Recombinant Pem-CMG

The hemolymph glucose level increased by 5 to 10 mg/dl after injection of 1 pair-equivalent of eyestalk extract into eyestalk-ablated prawns, whereas injection of PBS had little effect on glucose level in the hemolymph (Figure 4, A and B). Recombinant Pem-CMG, in both crude and purified form, had the ability to elevate hemolymph glucose levels of

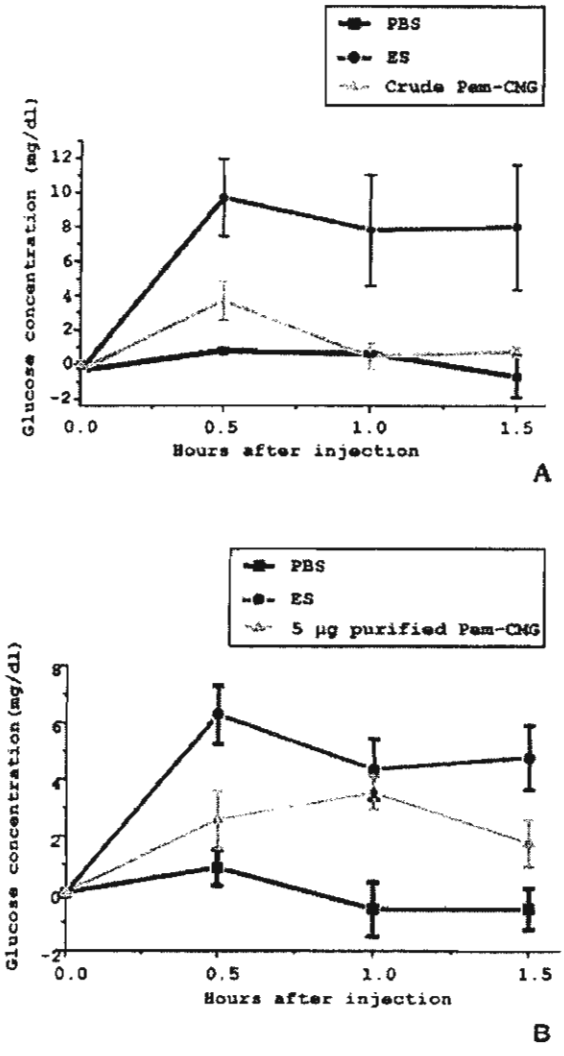

Figure 4. Determination of hyperglycemia in bilaterally eyestalk-ablated *P. monodon* after injection with crude recombinant Pem-CMG (**A**) or with purified recombinant Pem-CMG (**B**). The y-axis represents the glucose concentration (mg/dl) and the x-axis represents time at 0, 0.5, 1.0, 1.5 and 2 hours after injection. The level of the hemolymph glucose after injection with PBS (■), eyestalk extract (●), and crude Pem-CMG, after subtraction of the background glucose level from pPICZαA in A or purified Pem-CMG in B (▲), was determined every 0.5 hour. The error bars represent the SEM value ($n = 5$ and $n = 6$ for A and B, respectively).

eyestalk-ablated prawns, although to a smaller extent than compared with the crude eyestalk extract. The hyperglycemic effect could be observed from 0.5 hour, after injection. The highest levels of 3.5 and 3.25 mg/dl of hemolymph glucose were detected at 0.5 hour and 1 hour after injection with crude and purified recombinant Pem-CMG, respectively.

Discussion

The recombinant Pem-CMG was successfully expressed as a secreted peptide in *P. pastoris*. The result of amino-terminal sequencing showed amino acid sequence identical to that deduced from the cDNA encoding the mature Pem-CMG peptide (Udomkit et al., 2000). This suggests that the amino-terminus of the recombinant Pem-CMG was correctly processed by the KEX2 enzyme regardless of the absence of the double Glu-Ala repeats downstream of its cleavage site. The Glu-Ala repeat was suggested to be necessary for efficient cleavage by KEX2 (Cregg, 1999). The Glu-Ala residues left at the amino-terminus of the secreted peptide after Kex2 cleavage will later be removed by a dipeptidyl aminopeptidase encoded by the *STE13* gene (Clare et al., 1991; Wagner et al., 1992; Cregg et al., 1993; Van Nostrand et al., 1994; Raemaekers et al., 1999). However, our result demonstrates an example in which the Glu-Ala repeats are not necessary for KEX2 cleavage. A similar result was obtained with the expression of ASP2 in *P. pastoris* (Briand et al., 1999).

As determined by RP-HPLC, the acidic conditions in the purification step did not seem to affect the structure, and hence the biological activity, of the recombinant Pem-CMG, as the protein still retained activity after purification. The same strategy was successful in purification of CHH (Sithigorngul et al., 1999) and MIH (Ohira et al., 1999) in other species. It is possible that these peptides can tolerate a denaturing environment because their structures are held by 3 disulfide bonds that help stabilize the proteins.

Although the yield of the purified recombinant Pem-CMG expressed in this study was lower than that of other crustacean recombinant eyestalk neurohormones expressed in *E. coli* (Ohira et al., 1999; Gu et al., 2001), the recombinant Pem-CMG could be purified by one step of RP-HPLC, making the solubilization and renaturation steps required for obtaining active proteins expressed in *E. coli* unnecessary.

The ability to elevate the glucose level in the hemolymph of eyestalk-ablated *P. monodon* suggests that the recombinant Pem-CMG induces hyperglycemia, and thus Pem-CMG functions as CHH in *P. monodon*. This is in agreement with its primary structure—i.e., the lack of the amino acid glycine at position 12 of the mature peptide compared with those of MIH and GIH and the presence of an amidation signal, GK at the carboxy-terminal, which are characteristics of most CHH characterized so

far (Chang, 1997). The modification of translated proteins by amidation reaction is widespread in invertebrates and vertebrates but seems to be absent from yeast, as has been shown in *Saccharomyces cerevisiae* (Rourke et al., 1997). The recombinant Pem-CMG in this study may have different feature from the natural peptide in that it does not have the amidated carboxyl-terminus because of the possible lack of amidation in *P. pastoris*. The carboxy-terminal amidation could be essential for biological activity of proteins (Martinez et al., 1986); Therefore the lack of amidation may account for the low hyperglycemic activity of the recombinant Pem-CMG compared with that of natural substance in the eyestalk extract.

Although the major function of CHH is to regulate glucose metabolism in crustaceans, several studies have indicated that it may also contribute to the regulation of reproduction and the molting cycle (Chang et al., 1990; De Kleijn et al., 1995; Gu et al., 2000). Therefore the possibility that Pem-CMG also serves other functions in prawns cannot be excluded.

As shown in this study, yeast recombinant technology can produce biologically active proteins with high yield. As such, the recombinant crustacean eyestalk hormone is more desirable for further studies on topics such as structure-function relationships than are the native hormones that require large numbers of animals and complicated purification steps in the preparation process.

In summary, we have achieved expression of a biologically active eyestalk neuropeptide of *P. monodon* using the *P. pastoris* expression system. The recombinant Pem-CMG protein was characterized as a hyperglycemic hormone. As Pem-CMG is the first eyestalk hormone with a defined hyperglycemic activity that has been demonstrated in *P. monodon*, we propose that it be referred to as Pem-CHH1.

Acknowledgments

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Crustacean hyperglycemic hormones of *Penaeus monodon*: cloning, production of active recombinant hormones and their expression in various shrimp tissues

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Abstract

Crustacean hyperglycemic hormone (CHH) is the most abundant peptide in the eyestalk of crustacean. This hormone not only plays its major role in controlling glucose level in the haemolymph, but is also significant to other processes such as ecdysteroid synthesis and ovarian maturation. Multiple forms of CHH have been reported in several species. In addition to the Pem-CHH1 of *Penaeus monodon* recently identified, here, we report the cloning and characterization of the cDNA encoding another two Pem-CHH peptides, so-called Pem-CHH2 and Pem-CHH3. Both cDNAs contained 381-bp open reading frame encoding 127 amino acids. The cleavage at the putative processing site of the signal peptide, KR, would generate a 74 amino acids mature hormone for both Pem-CHH2 and Pem-CHH3. Amino acid sequence analysis revealed that Pem-CHH2 and Pem-CHH3 shared 95% identity in their amino acid sequences to that of Pem-CHH1. Both recombinant Pem-CHH2 and recombinant Pem-CHH3 expressed as secreted proteins in *Pichia pastoris* exhibited hyperglycemic activity at the comparable level to that of Pem-CHH1. Furthermore, investigation of the transcripts of each Pem-CHH in several tissues by RT-PCR showed that expression of *Pem-CHH1*, *Pem-CHH2* and *Pem-CHH3* was not restricted only to the eyestalk but also detectable in the heart. In addition, the transcript of Pem-CHH1 was also present in the gill. CHHs from various origins may play different roles and thus contribute towards its pleiotropic nature.

Keywords: cDNA; Crustacean hyperglycemic hormone (CHH); Expression; *Penaeus monodon*; *Pichia pastoris*

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1. Introduction

The eyestalk of decapod crustaceans contains neurosecretory neurons, from which a variety of peptide hormones is synthesized and secreted (Keller, 1992; Charmantier et al., 1997). 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 (MO-IH) (Wainwright et al., 1996; Chang, 1997). In addition to its major role in blood glucose regulation (Cooke and Sullivan, 1982), CHH is also involved in the regulation of several physiological processes such as edysteroid production (Yasuda et al., 1994), lipid metabolism (Santos et al., 1997), ovarian physiology (Khayat et al., 1998) and osmoregulation (Charmantier-Daures et al., 1994; Serrano et al., 2003).

CHH has been isolated from several crustaceans such as crabs (Kegel et al., 1989; Chung et al., 1998), lobster (Tensen et al., 1991), crayfishes (Kegel et al., 1991; Huberman et al., 1993), prawn (Sithigorngul et al., 1999) as well as isopod (Martin et al., 1984). CHH-related peptides were also found in insects. For instance, the amino acid sequence of the ion transport peptide of the locust, *Schistocerca gregaria*, was reported and revealed similarity to that of CHH (Meredith et al., 1995), and a cDNA encoding a CHH-family peptide was recently isolated from the silkworm *Bombyx mori* (Endo et al., 2000).

CHH exists as multiple isoforms in several of the species examined so far. Two isoforms of CHH were reported in *Homarus americanus* (De Kleijn et al., 1995), *Jasus lalandii* (Marco et al., 2000), *Orconectes limosus* (De Kleijn et al., 1994) and *Procambarus clarkii* (Yasuda et al., 1994), whereas at least five peptides with hyperglycemic activity were reported in *Penaeus japonicus* (Yang et al., 1997).

In *Penaeus monodon*, a cDNA encoding CHH has been identified and characterized (Udomkit et al., 2000). The recombinant protein expressed from this cDNA showed the ability to elevate glucose level in the haemolymph of eyestalk-ablated *P. monodon*, and thus designated Pem-CHH1 (Treerattrakool et al., in press). In this study, another two Pem-CHH cDNAs were isolated from the eyestalk of *P. monodon*. The biological activity of the recombinant proteins expressed from these cDNAs was determined and their expression in different tissues of *P. monodon* was also investigated.

2. Materials and methods

2.1. RNA extraction and cDNA cloning

Total RNA was isolated from a pair of eyestalks of a single shrimp. After removal of the cuticle and non-neural tissues, the dissected eyestalks were homogenized in TRIZOL Reagent (GIBCO BRL), and the RNA was extracted according to the manufacturer's protocol.

Rapid amplification of cDNA ends (RACE) technique was used to amplify both the 3′ and the 5′ regions of cDNA. The detailed protocols described earlier (Udomkit et al., 2000) were followed except that about 5 μg of total RNA were used as a template for first strand

cDNA synthesis instead of poly(A)$^+$RNA. The degenerate oligonucleotide PM2 was used as a gene specific primer in 3′ RACE for both *Pem-CHH2* and *Pem-CHH3* cDNAs. For 5′ RACE, CHH2-CSP1 and CHH3-CSP1 were used to prime first strand cDNA synthesis of *Pem-CHH2* and *Pem-CHH3*, respectively. Another two specific primers CHH2-CSP2 and CHH2-CSP3 were used for amplification of 5′ *Pem-CHH2* cDNA, whereas CHH3-CSP2 and CHH3-CSP3 were used for 5′ *Pem-CHH3* cDNA.

The coding regions of the cDNAs were amplified by RT-PCR using primers CDF-2 and CDR for *Pem-CHH2* and primers CDF-3 and CDR for *Pem-CHH3*.

The nucleotide sequences of the primers used in these experiments are shown in Table 1.

2.2. DNA sequence analysis

The PCR products were either cloned into pUC18 at specific sites or directly cloned into pGEM®-T Easy vector (Promega). Nucleotide sequences were determined by the method of ABI™ PRISM Dye Terminator Cycle (PE Applied Biosystems).

2.3. Protein expression and purification

The cDNA encoding the mature Pem-CHH2 peptide was amplified with primers CHH2-Ex and CDR-2. The amplified fragment was digested with *Sal*I and *Xba*I before ligating to *Pichia pastoris* expression vector pPICZαA (Invitrogen) that had been digested with *Xho*I and *Xba*I. The cDNA encoding the mature Pem-CHH3 peptide was amplified with primers CHH3-Ex and CDR-2, then subcloned into pPICZαA using the same

t1.1 Table 1
t1.2 Primers used for cDNA cloning

		Name	Sequence (5′ → 3′)	Size	T_m (°C)
t1.4	3′RACE	PM2	CCGGAATTCTGYGAAGAYTGYTACAAC	27	70
t1.5	5′RACE				
t1.6	Pem-CHH2	CHH2-CSP1	CCGGAATTCCCTTTGACGAGGCCGGAAC	28	90
t1.7		CHH2-CSP2	CCGAAGCTTGTCCACGCAGTAGAG	24	76
t1.8		CHH2-CSP3	CCGGAATTCACCTCGTTGTGGAAACAG	27	82
t1.9	Pem-CHH3	CHH3-CSP1	ATGCTTTATGAAGACATTAC	20	52
t1.10		CHH3-CSP2	CGTCAATATGGTGTCAGC	18	54
t1.11		CHH3-CSP3	GCAAGCTTCCTAGAGTCTGGTCATTC	26	58
t1.12	Coding region				
t1.13	Pem-CHH2	CDF-2	CGGGATCCATGGTTGCGGTCCGATTGG	27	82
t1.14		CDR	CGGGATCCCTACTTGCCGAGCCTCTA	26	77
t1.15	Pem-CHH3	CDF-3	GGCATATGGTTGCGGTCCGATTGG	24	66
t1.16	Protein expression				
t1.17	Pem-CHH2	CHH2-Ex	ATGAATTCGTCGACAAAAGATCTATATG GTTCAATTC	37	65
t1.18		CDR-2	GCTCTAGACTACTTGCCGAGCCTCTG	26	60
t1.19	Pem-CHH3	CHH3-Ex	ATGAATTCGTCGACAAAAGAACCATGTC GTTCAGTTC	37	71

strategy The physical maps of the resulting plasmids, αCHH2 and αCHH3, are shown in Fig. 1.

An overnight culture of *Pichia* transformants was transferred into 100 ml of fresh BMGY medium [1% (w/v) yeast extract, 2% (w/v) peptone, 0.67% (w/v) YNB, 4 μg/ml D-biotin, 100 mM potassium phosphate, pH 6.0 and 1% (v/v) glycerol] and was grown at 30 °C with shaking at 250 rpm until the OD_{600} reached 5–6. The cell pellet was then collected and resuspended in 1/5 volume of BMMY medium [1% (w/v) yeast extract, 2% (w/v) peptone, 0.67% (w/v) YNB, 4 μg/ml D-biotin, 100 mM potassium phosphate]. The culture was added with methanol at various concentrations from 0% to 5% (v/v) before incubated at the same condition. An aliquot of the culture was collected daily from 0 to 7 days. After the cells were discarded, the culture medium was subjected to further analysis for Pem-CHH2 and Pem-CHH3 expression.

The recombinant Pem-CHHs expressed and secreted into the culture medium were partially purified by ammonium sulfate precipitation prior to purification using Bplc® system (Amersham Pharmacia Biotech). The protein sample was loaded to a Superdex 75 PC 3.2/30 column. The column was eluted with two-column volume of phosphate buffer saline (PBS) pH 7.4 at the flow rate of 0.4 ml/min. The eluted fractions were collected every 1 ml by the fraction collector (Frac-900, Amersham Pharmacia Biotech), and were vacuum-dried before dissolved in PBS, pH7.4 and used for biological assay. The amount of proteins was determined by Bradford's method (Bradford, 1976) using the Bio-Rad Protein Assay kit.

FPLC

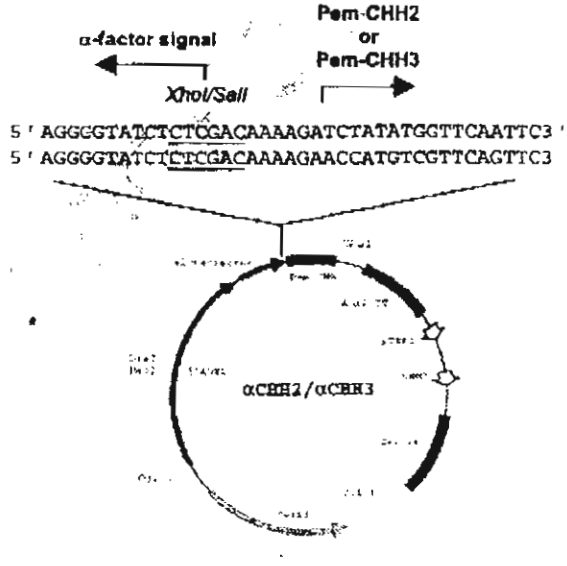

Fig. 1. The physical map of αCHH2 and αCHH3 expression plasmids. The *SalI/XbaI* digested cDNA fragments encoding the mature Pem-CHH2 and Pem-CHH3 were inserted into pPICZαA vector at *XhoI* and *XbaI* sites. This insertion allows the mature Pem-CHH2 and Pem-CHH3 cDNAs to fuse in-frame with the α factor secretion signal. The expanded region shows the nucleotide sequence of the junction at which the α factor secretion signal is followed by the coding sequence for the dibasic KR cleavage site (AAAAGA), which precedes the cDNA for the mature Pem-CHH2 and Pem-CHH3

2.4. Biological assay for hyperglycemic activity

Farm grown black tiger shrimp, *P. monodon* (~ 20 g), were bilaterally eyestalk ablated and kept in tanks filled with seawater (approximately 11 ppt of salinity) for 18 h with starvation. About 50 μl of the haemolymph were collected from individual shrimps for the measurement of baseline glucose level. Five micrograms of either the purified Pem-CHH 2 or the purified Pem-CHH 3 were then injected into 10 individual shrimps through the arthrodial membrane of the second walking leg. For the negative and positive control groups, each individual prawn was injected with 100 μl of PBS and one pair equivalent of eyestalk's neuron crude extract, respectively. The glucose levels in the haemolymph of individual shrimps were determined at 0.5, 1 and 1.5 h after injection using a glucose diagnostic kit (Sigma).

2.5. Dot-blot hybridization

Screening for the recombinants by dot-blot hybridization was conducted as follows. One microliter of 10 pg of each DNA sample was heat-denatured in boiling water for 5 min, snap cooled on ice and dotted on the GeneScreen Plus® hybridization transfer membrane (Dupont). The hybridization was performed as described below.

The DNA probes were synthesized by incorporation of Fluorescein-11-dUTP with the Gene Image random prime labelling module (Amersham Pharmacia Biotech) following the manufacturer's protocol. Hybridization was performed in hybridization solution [5 × SSC, 0.1% (w/v) SDS, 5% (w/v), dextran sulphate, 20-fold dilution of liquid block and 100 μg/ml denatured salmon sperm DNA] at 65 °C overnight. After hybridization, the first wash was performed in 1 × SSC, 0.1% (w/v) SDS at 65 °C for 15 min, followed by the second wash in 0.2% SDS, 0.1% (w/v) SDS at the same temperature for 15 min. The detection step was conducted according to the protocol of the Gene Images CDP-Star detection module (Amersham Pharmacia Biotech).

2.6. Reverse transcription polymerase chain reaction (RT-PCR) of Pem-CHH transcripts

Total RNA extracted from several tissues of *P. monodon* including eyestalk, gill, heart, muscle and thoracic ganglia was used as a template for RT-PCR. The reverse transcription step was performed with Impromt-II(tm)™ Reverse Transcriptase (Promega) according to the manufacturer's protocol using PRT1 primer (5′-CCGGAATTCAAGCTTCTAGAG-GATCCTTTTTTTTTTTTTTT-3′) to prime cDNA synthesis. The amplification of cDNA was performed with CMG-F primer (5′-CGGAATTCTCAGTGCAGAGGGA-GAGCC-3′) and PM1 primer (5′-CCGGAATTCAAGCTTCTAGAGGATCC-3′). The product from the first round of amplification with CMG-F and PM1 primers was used as a template for specific amplification of *Pem-CHH1* and *Pem-CHH3* cDNAs using the following pairs of primers: α-CMG (5′-ATGAATTCGTCGACAAAAGAAGCC-TATCCTTCAGGTC-3′) and CDR-2 for *Pem-CHH1* and CHH3-Ex and CDR-2 for *Pem-CHH3*. Specific amplification of *Pem-CHH2* cDNA was performed using CMG-F and CHH2-R (5′-GCGGATCCCTGCTTTATGAAGACACTG-3′) in the first round and using CHH2-Ex and CDR-2 primers in the second round. The amplification was achieved

by 35 successive cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min followed by a final extension at 72 °C for 7 min.

3. Results

3.1. Cloning of the cDNA encoding Pem-CHH2 and Pem-CHH3 peptides

The PCR products from 3'RACE were purified and cloned into pUC18 vector at *Eco*RI site. A total of 130 transformants were firstly screened by DNA dot-blot hybridization using a 384-bp open reading frame of *Pem-CHH1*, previously called *Pem-CMG* (Udomkit et al., 2000) as a probe. Five recombinant clones producing positive hybridization signal were subjected to subsequent analysis by DNA sequencing. The nucleotide sequences of these five 3'-cDNAs were aligned using Clustalx program. Three of these 3' cDNA showed 97–99% identity in their nucleotide sequences to the cDNA encoding hyperglycemic hormone homolog of *P. monodon*, PmSGP-IV (AF104389), PmSGP-II (AF104387) and PmSGP-III (AF104388), previously submitted to the Genbank database by Davey et al. Whereas the other two clones, designated 3'-*CHH2* and 3'-*CHH3*, shared around 72% nucleotide identity to the cDNA encoding Pem-CHH1 (AF233295) (data not shown). Using a set of specific primers derived from the 3'-*CHH2* and 3'-*CHH3* cDNA, the corresponding 5' fragments were successfully cloned. The combination of the nucleotide sequences between the 3' and 5' fragments gave rise to the complete *Pem-CHH2* and *Pem-CHH3* cDNA. The nucleotide and deduced amino acid sequences of *Pem-CHH2* and *Pem-CHH3* are shown in Fig. 2.

3.2. Nucleotide sequence analysis of Pem-CHH2 and Pem-CHH3 cDNA

Pem-CHH2 cDNA contained a 381-bp open reading frame encoding 127 amino acids. A cleavage at the putative processing site (KR) of Pem-CHH2 would generate a 53 amino acids signal peptide and a mature peptide of 74 amino acid residues. A 381-bp open reading frame of *Pem-CHH3* also coded for 127 amino acid residues. Pem-CHH3 contained a putative mature peptide of the same length as that of Pem-CHH2. The alignment of the amino acid sequences of the putative mature Pem-CHH2 and Pem-CHH3 peptides with that of the previously characterized Pem-CHH1 in Fig. 3 revealed that their mature peptides shared high level of similarity (95%). The three Pem-CHH peptides also exhibited a unique characteristic of the CHH/MIH/GIH family, i.e., the alignment of the six cysteine residues at identical positions.

3.3. Expression of recombinant Pem-CHH2 and Pem-CHH3 in P. pastoris

Pem-CHH2 and *Pem-CHH3* cDNA were separately cloned into pPICZαA vector to produce αCHH2 and αCHH3 recombinant plasmids, respectively (Fig. 1). The recombinant plasmids were later introduced into *P. pastoris* KM71. The transformants having either αCHH2 or αCHH3 integrated into their genome were examined by PCR. The

A

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tcagtgcagagggagagcctcgaagtcggtggtccttgttccttctgctctctgtcgagt    60
ccagcgaaatggttgcggtccgattggtgcagtcagctgttcttgtgtccttgctggtgg   120
         M  V  A  V  R  L  V  Q  S  A  V  L  V  S  L  L  V
cacttccggcctgtgtcacaacttctgaaaacacgaatgaaatacccgcgtccattcttt   180
A  L  P  A  C  V  T  T  S  E  N  T  N  E  I  P  A  S  I  L
cctccctgtgggattcccttcagaagaccaaagcataagcaaacgctctatatggttca   240
S  S  P  G  D  S  L  S  E  D  Q  S  I  S  K  R  S  I  W  F
attcttgcacgggcgtctacgaccgcgaacttcttgtaaggctcgaccgcgtgtgcgaag   300
N  S  C  T  G  V  Y  D  R  E  L  L  V  R  L  D  R  V  C  E
actgctacaacctctaccgcgacgtcggagtggcggccgaatgcaggagtaactgtttcc   360
D  C  Y  N  L  Y  R  D  V  G  V  A  A  E  C  R  S  N  C  F
acaacgaggtgttcctgtactgcgtggactacatgttccggcctcgccagaggaaccagt   420
H  N  E  V  F  L  Y  C  V  D  Y  M  F  R  P  R  Q  R  N  Q
accgggccgcccctccagaggctcggcaagtaggcggctcgtcttcggtcaacccttcctg   480
Y  R  A  A  L  Q  R  L  G  K  *
cggggacgctcgccgtgagatttttctaggctctccttcggttgcttctgtatcctaag    540
ttagaaacggataacatattgacgtcgaggtgtttcatgtacagtgtcttcataaagcag    600
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B

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tcagtgcaggggagagcctcgaagtcggtggtccttgttcccgctgatcgttgcgagt     60
ccagcgaaatgattgcggtccgactggtgcagtcagctgttctggtgtccctgctggtgg   120
         M  I  A  V  R  L  V  Q  S  A  V  L  V  S  L  L  V
catttccggcctgtgtcacgacttctgaaaacacgaatgaaatactggcgtccattcttt   180
A  F  P  A  C  V  T  T  S  E  N  T  N  E  I  L  A  S  I  L
cctccctggggattcccttacacgagaacaaagcataagcaaacgcaccatgtcgttca   240
S  S  P  G  D  S  L  T  R  E  Q  S  I  S  K  R  T  M  S  F
gttcttgcacgggtgtctacgaccgcgaactccttgtaaggctcgaccgcgtgtgcgaag   300
S  S  C  T  G  V  Y  D  R  E  L  L  V  R  L  D  R  V  C  E
actgctacaacctctaccgcgacgttggagtggcggccgaatgtaggagtaactgtttcc   360
D  C  Y  N  L  Y  R  D  V  G  V  A  A  E  C  R  S  N  C  F
acaacgaggtgttcctgtactgcgtggactacatgtttcggcctcgccaaaggaatcagt   420
H  N  E  V  F  L  Y  C  V  D  Y  M  F  R  P  R  Q  R  N  Q
accgggccgccctgcagaggctcggcaagtaggcggctcctatccggccagctcttccgc   480
Y  R  A  A  L  Q  R  L  G  K  *
acgggcgctcgctgtgagactttctgaatgaccagactctaggctctcttcggttgcctc   540
tgtatcctaagttagaagctgacaccatattgacgccgaggtgtttcatgtgtaatgtct   600
tcataaagcat                                                    611
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Fig. 2. The nucleotide and deduced amino acid sequences of Pem-CHH2 (A) and Pem-CHH3 (B). Amino acids are shown in one-letter symbol. Asterisk marks the stop codon. The putative cleavage site, KR, is boxed in both sequences.

optimal condition for expression was induction with 3% (v/v) methanol for 2 days for Pem-CHH2 and induction with 4% (v/v) methanol for 2 days for Pem-CHH3. An SDS-PAGE analysis of the culture supernatant of both αCHH2 and αCHH3 *P. pastoris* transformants showed major protein products of about 8 kDa, which were of the expected size for Pem-CHH2 and Pem-CHH3 (lane 1 in Fig. 4A,B, respectively). These protein bands were not present in the culture supernatant of *P. pastoris* transformant containing pPICZαA vector alone (data not shown). Purification of these secreted products by size exclusion could remove all contaminated proteins from Pem-CHH2 and Pem-CHH3 as shown by SDS-PAGE in lane 2, in Fig. 4A,B, respectively. The final yield of the purified Pem-CHH2 and Pem-CHH3 was 270 and 450 μg/l of the culture medium, respectively.

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                        *          20         *          40         *
Pem-CHH1  :  SLSFRSCTCAYDRELLVRLDRVCEDCYNLYRDVGVAAECRSNCFHNEVFL  :  50
Pem-CHH2  :  SIWFNSCTGVYDRELLVRLDRVCEDCYNLYRDVGVAAECRSNCFHNEVFL  :  50
Pem-CHH3  :  TMSFNSCTGVYDRELLVKLDRVCEDCYNLYRDVGVAASCRSNCFHNEVFL  :  50
Pm-SGP-I  :  SLFDRSCTGVFDRQLLRRLSRVCSDCFNVGREFRVATSCRSNCYYNEVFR  :  50
Pm-SGP-II :  ANFDESCACVYNRELLGRLSRLCDDCYNVFREFKVATECRSNCFYNPVFV  :  50
Pm-SGP-III:  ANFDESCACVYNRELLGRLSRLCDDCYNVFREFKVATECRSNCFLNPVFV  :  50
Pm-SGP-IV :  SLFDPACTGIYDRQLIGKLGRLCDDCYNVFREFKVATGCRSNCYYNLIPL  :  50
Pm-SGP-V  :  ANFDPSCAGVYDRELIGGLSRLCDDCYNVFREFRVATECRSNCFYNSVFV  :  50

                        60         *
Pem-CHH1  :  YCVDYMFRPRQRNCQRALQRLGK  :  74
Pem-CHH2  :  YCVDYMFRPRQRNCQRALQRLGK  :  74
Pem-CHH3  :  YCVDYMFRPRQRNCQRALQRLGK  :  74
Pm-SGP-I  :  QCMEYLIPAHLHEEHRLAVQMGK  :  74
Pm-SGP-II :  QCLEYLIPADLHEEVQRHVQTVGK  :  74
Pm-SGP-III:  QCLEYLIPADLHEELQRHVQTVGK  :  74
Pm-SGP-IV :  DCLEYLIPSHLQEEHMEALQTVGK  :  74
Pm-SGP-V  :  QCLEYLIPADLHEEVQRHVQTVGK  :  72
```

Fig. 3. An alignment of amino acid sequences of Pem-CHH1 to 3 (Udomkit et al., 2000 and this study) and Pm-sgp-I to -V (Davey et al., 2000). The amino acid residues that are identical in all sequences are highlight in black. The grey and light-grey color highlight the amino acid residues that are identical in six or seven and five sequences, respectively. The amino acid block MFRPRQRNQ presented only in the sequences of Pem-CHHs is boxed.

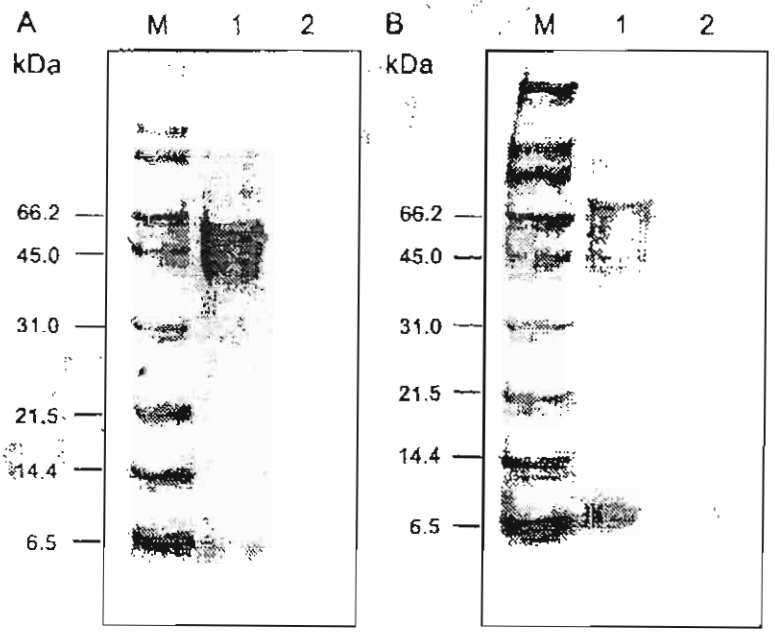

Fig. 4. Tricine SDS-PAGE analysis for expression of Pem-CHH2 (A) and Pem-CHH3 (B) in *P. pastoris*. Lane M represents broad range protein marker (Bio-Rad, USA) as a molecular weight standard. Lane 1 represents 100 μl of culture supernatant of *Pichia* transformant carrying *Pem-CHH2* cDNA induced with 3% (v/v) methanol for 2 days (A) and of *Pichia* transformant carrying *Pem-CHH3* cDNA induced with 4% (v/v) methanol for 2 days (B). Lane 2 in (A) and (B) represents 0.5 μg of Pem-CHH2 and Pem-CHH3 after purification, respectively.

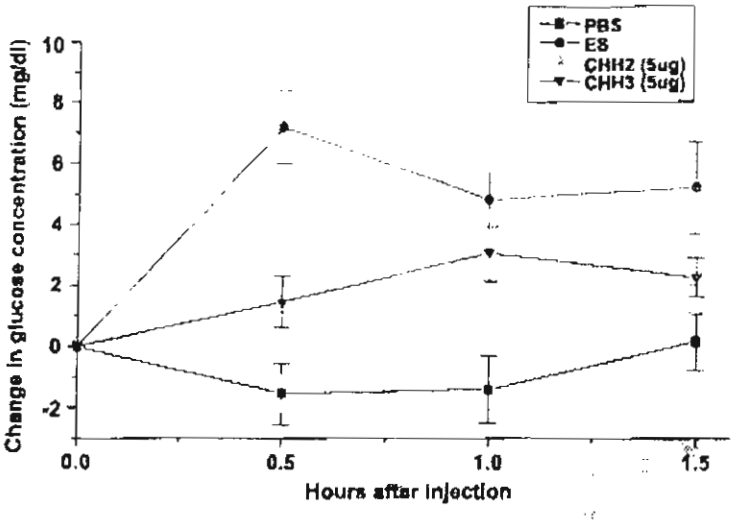

Fig. 5. Determination of glucose level in the hemolymph of eyestalk-ablated *P. monodon* after injection with 100 μl each of PBS (–■–), crude extract from one pair of eyestalk (–●–), 5 μg of purified Pem-CHH2 (–▲–) and 5 μg of purified Pem-CHH3 (–▼–). The hyperglycemia was presented as a change in glucose concentration (mg/dl) at each time points comparing to those at 0 h (before injection) for each group.

3.4. Hyperglycemic activity of recombinant Pem-CHH2 and Pem-CHH3

The hyperglycemic effect in the haemolymph of eyestalk-ablated *P. monodon* could be observed within 0.5 h after injection with both recombinant Pem-CHH2 and recombinant Pem-CHH3 (Fig. 5). Although the highest levels of haemolymph glucose (3.58 and 3.05 mg/dl for shrimp injected with Pem-CHH2 and Pem-CHH3, respectively) were somewhat lower than the level raised by injection of extract from eyestalk's neurons, they were

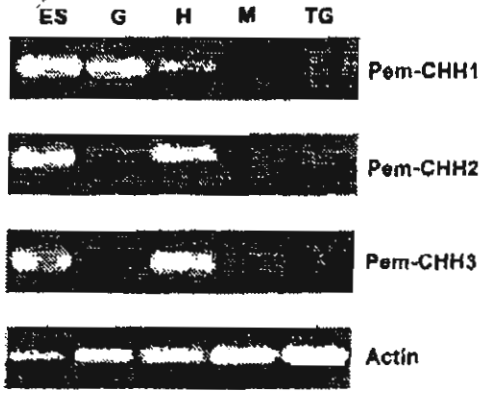

Fig. 6. Expression of *Pem-CHH* transcripts in different tissues of *P. monodon*. The RT-PCR products for Pem-CHH1, Pem-CHH2, Pem-CHH3 and β-actin were analyzed on 1.5% agarose gel. RNA from eyestalk, gill, heart, muscle and thoracic ganglia was a template for RT-PCR in the lanes indicated ES, G, H, M and TG, respectively.

significantly higher than that of the PBS-injected shrimps and, therefore, demonstrated the hyperglycemic activity of both Pem-CHH2 and Pem-CHH3.

3.5. Expression of Pem-CHHs in P. monodon

The transcripts of each *Pem-CHH* gene in *P. monodon* were examined in several tissues by RT-PCR. The RT-PCR products of *Pem-CHH1*, *Pem-CHH2* and *Pem-CHH3* were detected in both the eyestalk and the heart. These transcripts were not present in other tissues, i.e., gill, muscle and thoracic ganglia, except for the transcript of *Pem-CHH1* that was also detectable in the gill (Fig. 6).

4. Discussion

Multiple molecular forms of CHH have been reported in several species: two forms of CHH were identified in *H. americanus* (Tensen et al., 1991) and *Metapenaeus ensis* (Gu and Chan, 1998, Gu et al., 2000) and five peptides with hyperglycemic activity were reported in *P. japonicus* (Yang et al., 1997). In *P. monodon*, the first CHH, namely Pem-CHH1, has been described (Udomkit et al., 2000). Another five CHH-homologs in *P. monodon* (Pm-sgp-I to -V) were also identified by Davey et al. (2000). These suggest that CHHs are also encoded in multiple forms in *P. monodon* as in other species. Here, we reported two additional *P. monodon*'s CHHs, so-called Pem-CHH2 and Pem-CHH3. Both Pem-CHH2 and Pem-CHH3 share 60–70% similarity to CHH of other crustaceans. Most of the differences in the amino acid sequence among the three types of Pem-CHH lie within the first five amino acid residues at the N-terminus of their mature peptides. However, these Pem-CHHs can be distinguished from Pm-sgp-I to -V by the presence of a conserved amino acid block MFRPRQRNQ, residues 56–64, which can be found only in Pem-CHH1–3. This different feature suggests that Pem-CHH and Pm-sgp belong to different subgroups of the CHH in *P. monodon*.

Both Pem-CHH2 and Pem-CHH3 showed hyperglycemic activity through the capability to elevate the glucose level in the haemolymph of eyestalk ablated *P. monodon*. The hyperglycemia levels produced by Pem-CHH2 and Pem-CHH3 were comparable to that of Pem-CHH1. CHH is classified into type I of CHH family peptides (Lacombe et al., 1999). One of the distinctive characteristics of the hormones in type I is the amidated carboxyl-terminus (C-terminus). Recently, this carboxyl-terminal amide moiety has been shown to play significant role in conferring hyperglycemic activity of crustacean hyperglycemic hormone, Pej-SGP-I, in *P. japonicus* (Katayama et al., 2002). The recombinant Pej-SGP-I having a free C-terminus showed lower hyperglycemic activity by approximately one order of magnitude than that of the recombinant Pej-SGP-I with an amidated C-terminus. In our studies, the recombinant Pem-CHHs were expressed in *P. pastoris*, in which no information about amidation is available. However, it was demonstrated that the yeast *Saccharomyces cerevisiae* lacks the ability to amidate proteins (Rourke et al., 1997). It is, therefore, possible that the recombinant Pem-CHHs expressed in *P. pastoris* may have free C-terminus and, as a consequence, exhibited lower hyperglycemic activity than that of the natural hormones.

Although the eyestalk has long been known as a source for CHH production (Kleinholz et al., 1967), the results of RT-PCR showed that *Pem-CHH1*, *Pem-CHH2* and *Pem-CHH3* were also expressed in the heart. CHH-like peptides originated from pericardial organ neurosecretory cells, so-called PO-CHH, have been found in the crab *Carcinus maenas* (Direksen et al., 2001). The PO-CHH and the CHH from sinus gland in this species arise by alternative splicing of the same mRNA precursor. As a consequence, these two types of CHH have the same amino acid sequences in the N-terminal part but are different at the C-terminus. In addition, PO-CHH did not exhibit hyperglycemic activity. In this study, the nucleotide sequence of the Pem-CHH transcript from the heart is identical to that of the sinus gland-derived CHH (data not shown). However, physiological roles of CHH synthesized from the heart of *P. monodon* remain to be elucidated. Interestingly, the transcripts of *Pem-CHH1* were also detected in the gill. In the other experiments, the RT-PCR product from the gill was also observed for Pem-CHH3 (data not shown). Although there has been no report on the gill-associated CHH in other species so far, the CHH isolated form the sinus gland of the crab *Pachygrapsus marmoratus* was demonstrated to be involved in the control of gill ion transport (Spanings-Pierrot et al., 2000). It is possible that the CHH originated from the gill may be a major factor that contributes to the control of osmoregulation. However, its physiological role needs further investigation.

In summary, cDNAs encoding two additional CHH of *P. monodon*, so-called Pem-CHH2 and Pem-CHH3, were cloned and characterized. The recombinant proteins expressed from *Pem-CHH2* and *Pem-CHH3* cDNAs in *P. pastoris* were biologically active and exhibited hyperglycemic activity at a level comparable to that of Pem-CHH1. Furthermore, expression study of the three *Pem-CHH* genes in different tissues of *P. monodon* showed that transcription of *Pem-CHH1*, *Pem-CHH2* and *Pem-CHH3* was not only restricted to the eyestalk but also occurred in the gill and the heart. This result implies that CHH may play multi-functional roles and thus supports the evidences provided by several workers. Functional study of these tissue-specific CHHs will provide an inference for clearer understanding of the physiological roles of this pleiotropic hormone.

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O-Shrimp-08

Expression of growth-related peptide, Pem-CMG, of *Penaeus monodon* in methylotrophic yeast *Pichia pastoris*

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Abstract

Crustacean hyperglycemic hormone (CHH), molt-inhibiting hormone (MIH) and gonad-inhibiting hormone (GIH) are members of novel peptide family produced from the X-organ sinus gland complex in the eyestalk of crustaceans. This peptide family plays important roles in controlling several physiological processes such as regulation of growth and reproduction. In this study, the cDNA encoding a peptide related to the CHH/MIH/GIH family (so-called *Pem-CMG*) of the black tiger prawn, *Penaeus monodon*, was successfully expressed in the yeast *Pichia pastoris* by *AOX1* promotor. The recombinant Pem-CMG was secreted into the culture medium using α-factor signal sequence from *Saccharomyces cerevisiae* with an accumulated yield of 150 mg/l in the induction medium. The N-terminus of the recombinant Pem-CMG was determined and the result showed that it was correctly processed. The recombinant Pem-CMG was purified by one-step reverse phase HPLC and was used in biological assay for CHH activity. The recombinant Pem-CMG produced from *P. pastoris* demonstrated the ability to elevate glucose level in the haemolymph of eyestalk-ablated *P. monodon* suggesting that Pem-CMG possesses the activity of hyperglycemic hormone.

Introduction

The X-organ sinus gland (XOSG) complex located in the optic ganglia in the eyestalk is the major neuroendocrine control center of crustaceans. The major hormone family produced in the XOSG complex is composed of crustacean hyperglycemic hormone (CHH), molt-inhibiting hormone (MIH) and gonad-inhibiting hormone (GIH). Apart from the small quantity presented in nature, the similarity in size and the primary structure of this neuropeptide family limits the isolation and purification of these peptides directly from crustacean eyestalk. Recombinant DNA technology is one of the simplest and fastest methods to obtain large quantities of purified proteins. Recently, the

cDNA sequences encoding peptides in the CHH/MIH/GIH family in *P. monodon* were reported (Udomkit et al.,2000 and Davey et al.,2001. The recombinant protein from one of these cDNA sequences, Pem-CMG, was expressed in *E. coli* expression system (Chooluck, 1999). Unfortunately, The protein was expressed in an insoluble form. To overcome the problems of the complicating downstream processes such as solubilization and renaturation of the recombinant protien, *Pichia pastoris* was chosen to be used as expression system for Pem-CMG in this study because it is an ideal eukaryotic host cell for synthesizing a great deal of heterologous proteins. Moreover, it usually gives higher expression levels and less complicating downstream processes as it has a tendency to produce heterologous protein in a soluble form (Hitzman RA, 1981).

Materials and Methods

Construction of expression plasmid

A DNA fragment containing the coding sequence for mature Pem-CMG was obtained by PCR amplification from the plasmid Pem-CMG-EX2.1 (Chooluck, 1999). The amplified DNA fragment was then cloned into pPICZαA expression vector resulting in the recombinant plasmid αCMG, which contain the coding sequence for mature Pem-CMG fused in-framed with the α-factor secretion signal of *Saccharomyces serevisiae* without the double Glu-Ala repeats. The physical map of the recombinant plasmid αCMG was shown in figure 1.

Transformation of Pichia pastoris by electroporation

The recombinant plasmid αCMG was linearized by PmeI, and used for transformation of Pichia pastoris strain KM71 by electroporation with the condition: 1.5 kV. 25 μF and 200 Ω. After addition with 1M sorbitol, the cells were incubated at 30°C without shaking for 1 h, then YEPD was added and the incubation was continued with shaking at 30°C for 2 h. The cells were pelletted and resuspended in 100 μl sterile distilled water. The cell suspension was spread on YEPD plate containing 100 μg/ml Zeocin™, and was incubated for 2-3 days until colonies formed. The transformants were screened for integration of αCMG into the genome by PCR amplification with 5'AOX and 3' AOX primers.

Expression of recombinant αCMG

A single colony of P. pastoris KM71 recombinant containing αCMG was inoculated in 5 ml of YEPD and incubated at 30° C with shaking at 250 rpm for 48 h. Then, the cell culture was transferred into 100 ml of fresh BMGY medium and grown in the same condition until the culture reached an OD_{600} of 5 – 6. The cell pellet was harvested and resuspended in BMMY using 1/5 volume of the original culture. Absolute methanol was added to a final concentration of 3% (v/v) to maintain the induction for 3 days. The expressed recombinant Pem-CMG in the culture supernatant was analyzed on 16.5% Tricine SDS-PAGE and the amino acid sequence at the N-terminus was determined by N-terminal sequencing.

Purification of recombinant αCMG

After three days of induction, the culture supernatant was subjected to partial purification by precipitation with 30-50% ammonium sulfate and was concentrated by ultrafiltration using the Centriprep® centrifugal filter devices (amicon® bioseparations). The concentrated partially purified protein was further purified by reverse phase-HPLC on Jupiter Phenomenex C18 column (Phenomenex, 5 μ particle, 300°A pore size, 250 x 4.6 mm). The column was eluted with a linear gradient of 0-10% acetonitrile in 0.1% (v/v) trifluroacetic acid (TFA) for 1 min, holding at 10% acetonitrile in 0.1% (v/v) trifluroacetic acid (TFA) for 10 min, followed by a linear gradient of 10-60% acetonitrile in 0.1% (v/v) trifluroacetic acid (TFA) for 40 min, holding at 60% acetonitrile in 0.1% (v/v) trifluroacetic acid (TFA) for 10 min at a flow rate of 1 min/ml. The expected fraction was collected and then freeze dried. The purified protein was dissolved with PBS (137.9 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4.2H_2O$, 1.76 mM KH_2PO_4, pH7.4) and used for biological activity assay.

Biological assay for hyperglycemic activity of recombinant Pem-CMG

Shrimps (16-25 g) were cultured in the tanks filled with seawater (approximately 11 ppt of salinity) at the depth of 8-10 cm with good aeration for 3-4 h. Shrimps were then bilaterally eyestalk ablated with a pair of sharp scissors and the wounds were immediately sealed with hot forceps and returned to the culture conditions.

After bilaterally eyestalk ablated, the shrimps were fasted for 18 h. Ten shrimps were used for each group. One hundred microliters of hemolymph were removed from the individual shrimp for baseline measurement of glucose levels. Five micrograms of purified recombinant Pem-CMG in a volume of 100 μl was injected into an individual shrimp through the arthrodial membrane of the second walking leg by a syringe. A volume of 100 μl of PBS and two eyestalk-equivalent of crude extract was injected in each control group as negative and positive controls, respectively. After

injection, shrimps were returned to the culture tanks, and 100 μl of haemolymph of the individual shrimp was removed at 0, 0.5, 1 and 1.5 h for measurement of glucose level using the glucose diagnostic kit (Sigma, USA).

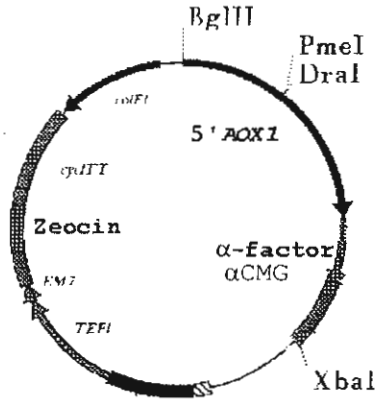


Figure 1. The Physical map of αCMG the recombinant αCMG plasmid contains *Pem-CMG*cDNA that fused in-frame with the α-factor secretion signal without Glu-Ala repeats, AOX1 promoter and Zeocin™ resistant gene.

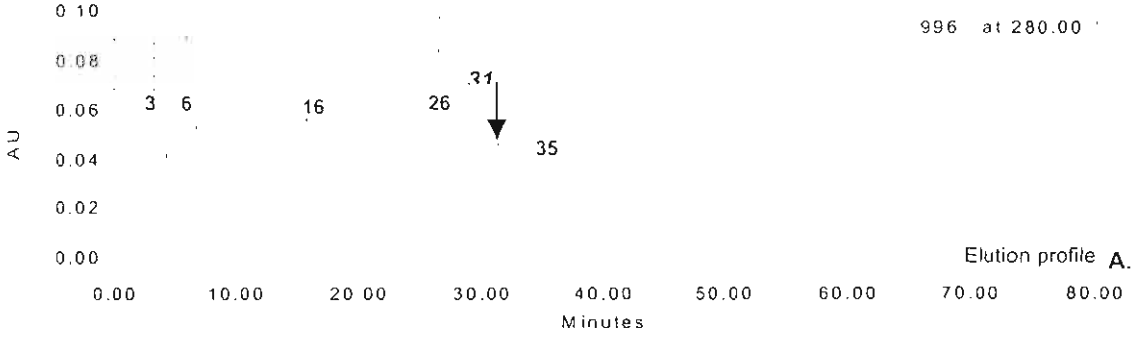
Results

Analysis of Pem-CMG expression in P. pastoris

An SDS-PAGE analysis of the culture supernatant of αCMG *P. pastoris* transformant showed the major protein product of about 8.3 kDa, which is the expected size for Pem-CMG (Figure 2, lene 1). This protein band was not present in the culture supernatant of *P. pastoris* transformant containing pPICZαA alone. The N-terminal sequencing of this protein showed that the first five amino acid residues were Ser-Leu-Ser-Phe-Arg corresponding to the deduced amino acid sequence from the Pem-CMG cDNA. The total yield of secreted proteins form *P. pastoris* transformant containing αCMG was 150 mg/l as determined by Bradford's method using Bio-Rad Protein Assay kit.

Purification of recombinant Pem-CMG

Partial purification by ammonium sulfate precipitation was successful in removing some impurities from the culture supernatant of *P. pastoris* transformant containing αCMG as shown in figure 2 (lane 2). The RP-HPLC elution profile of the partially purified Pem-CMG and the result of Tricine SDS-PAGE (Figure 2A and 2B) showed that the protein of the expected size for Pem-CMG was eluted at 31 minutes.



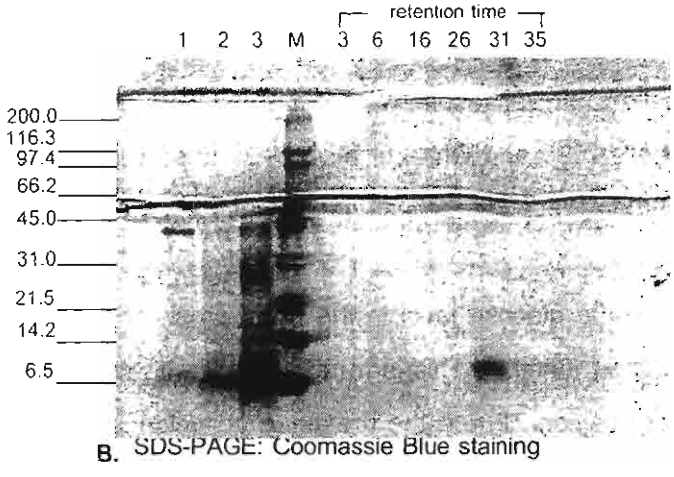


Figure 2. Purification of recombinant Pem-CMG Panel A shows the chromatogram pattern of partially purified recombinant Pem-CMG by HPLC observed a the wavelength of 280 nm. Panel B shows a Tricine SDS-PAGE of 3 µg of the culture supernatant (Lane 1), 3 µg of the partially purified Pem-CMG from 30-50% ammonium sulfate precipitation (Lane 2) 10 µg of the concentrated partially the purified by ultrafiltration (Lane 3) and the purified fractions o Pem-CMG by using HPLC at the retention time of 3, 6 16, 26, 31 and 35

Hyperglycemic activity of recombinant Pem-CMG

The result of an assay for hyperglycemic activity showed that injection of two eyestalk-equivalent protein extract of *P. monodon* raised the haemolymph glucose level of eyestalk-ablated *P. monodon* to about 5 mg/dl. The purified recombinant Pem-CMG also had an ability to elevate haemolymph glucose level after injection into eyestalk-ablated *P. monodon* to a level of about 3 mg/dl whereas injection of PBS had no effect on glucose level in the haemolymph (Figure 3).

Discussion

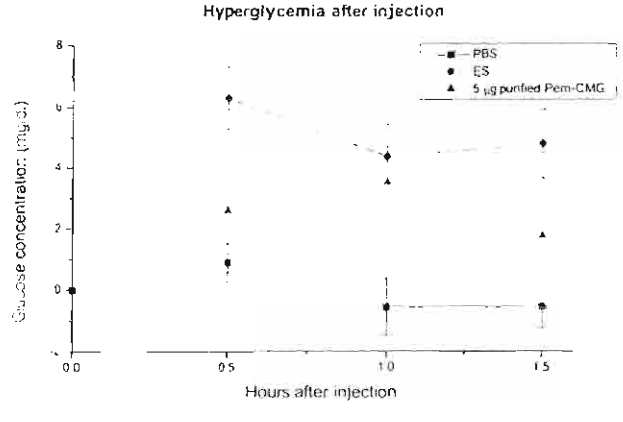


Figure 3. Biological assay of purified recombinant Pem-CMG in *P. monodon*. Effect of purified recombinant Pem-CMG on haemolymph hyperglycemic activity. The Y-axis in dicates the increase in haemolymph glucose level (mg/dl) and the X-axis represents the time of haemolymph sampling after 0, 0.5, 1.0 and 1.5 h

The recombinant Pem-CMG was successfully expressed as secreted peptide in *P. pastoris*. The result of N-terminal sequencing revealed that the N-terminus of the recombinant Pem-CMG was correctly processed by KEX2 enzyme regardless of the absence of the Glu-Ala repeats downstream of its cleavage site. Our result demonstrates an example in which the Glu-Ala repeats are not necessary for KEX2 cleavage. Similar result was obtained with the expression of ASP2 in *P. pastoris*. (Briand L et al., 1999).

Reverse phase HPLC was used for purification of Pem-CMG. The denaturing condition in the purification step did not seem to affect the structure, and hence, the biological activity of Pem-CMG as the protein was still active after purification. Similar results were observed with the purified CHH and MIH in other species (Sithigorngul et al., 1999 and Ohira et al., 1999). It is possible that the peptides can tolerate the denaturing environment because their structure was held by the three disulfide bonds that help stabilizing the proteins.

The recombinant Pem-CMG could elevate the glucose level in the hemolymph of eyestalk-ablated *P. monodon* suggesting that it possess CHH function. This is in agreement with its primary structure i.e. the lack the amino acid glycine at position 12 and the presence of amidation signal, GK at the C-terminus, which are the characteristics of most CHH characterized so far.

Although, CHH's major function is to regulate glucose metabolism, recent studies indicated that it might also play a role in the regulation of reproduction and molting cycle (De Kleijn et al., 1995, Chang ES et al., 1990 and Gu P-L et al., 2000). It is possible that Pem-CMG also serves other functions as MIH and GIH. Therefore, it is of interest to determine other functions of recombinant Pem-CMG peptide, including the ability of Pem-CMG to inhibit the release of ecdysone from Y-organ or vitellogensis in the ovary of *P. monodon*.

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Molecular cloning and expression of a putative molt-inhibiting hormone of *Penaeus monodon*.

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Abstract Growth in Crustacean occurs only when the exoskeleton is removed by a physiological process called molting. Molting is triggered by ecdysteriods that are synthesized and secreted from Y-organ. The synthesis of ecdysteroid is negatively regulated by molt-inhibiting hormone (MIH), a member belonging to the CHH/MIH/GIH peptide family of eyestalk neuropeptides produced by X-organ sinus gland complex (XOSG). In this study, a cDNA encoding the mature region of MIH of the black tiger prawn, *Penaeus monodon*, was obtained by amplification of first stranded cDNA from eyestalk using gene specific primers. This cDNA contained a 231 bp open reading frame encoding a 77 residues of putative mature MIH (Pem-MIH) that shows comparable degree of identity (94%) with the active MIH of *P. jaonicus*. Pem-MIH cDNA was inserted downstream of α-factor secretion signal of pPICZ αA *Pichia pastoris* expression vector under the control of *AOX1* promoter. The putative recombinant Pem-MIH, of expected size about 8.5 kDa, was expressed and secreted to culture medium approximately 5 mg/l after induced with 3% (v/v) methanol for 3 days.

Introduction

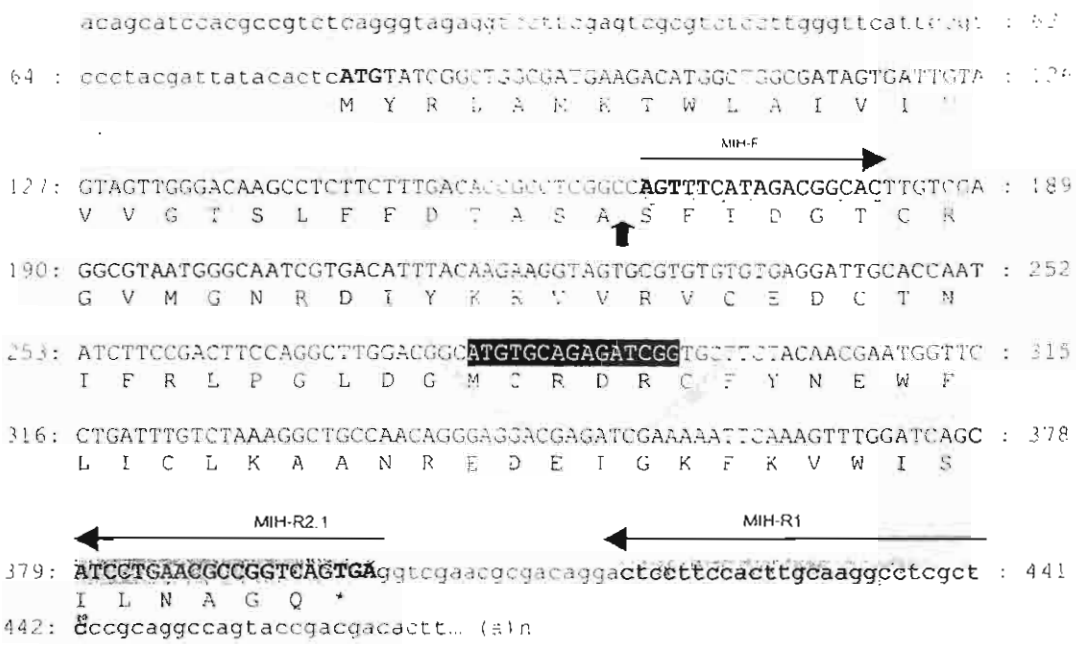
Molting is a physiological process that plays an important role to remove the old exoskeleton and allows growth in crustaceans to occur. This process is regulated by antagonistic function of two hormones. Ecdysteroid hormone, synthesized from Y-organ, stimulates molt. The activity of ecdysteroids is negatively regulated by the second hormone called molt-inhibiting hormone (MIH). MIH is a neuropeptide belonging to the CHH/MIH/GIH family that is produced by a group of neuroendocrine cells in the X-organ sinus gland complex (XOSG) located in eyestalks. The hormones in this family comprise crustacean hyperglycemic hormone (CHH), molt-inhibiting hormone (MIH) and gonad-inhibiting hormone (GIH) that are involved in blood sugar regulation, inhibition of ecdysteroid synthesis and regulation of reproduction, respectively (Chang, 1993; Huberman, 2000). These hormones consist of 72-90 amino acid residues of their mature peptide and contain six conserved cystein residues at identical positions.

In Thailand, Black tiger prawn (*Penaeus monodon*) is one of the most economically important species that is raised in numerous aquaculture farms along the coast line. One of the problems that most of the shrimp farms are encountering is growth retardation that can be related to molting process. Study of MIH will lead to understanding how the molting process is regulated. This could be the major contribution toward the improvement of black tiger prawn quality. Isolation and purification of MIH from crude eyestalk extracts have some limitations due to the similarity in size and primary structure of the peptides in this family. To avoid this problem, recombinant DNA technology has been employed in order to obtain a large quantity and higher purity of MIH. In 2001, Gu, P.L., et al. cloned a cDNA encoding MIH *of Metapenaeus ensis* (Mee-MIH) and expressed the recombinant Mee-MIH in *E. coli*. Although the bacterial expression system produced the recombinant protein at high level, this protein could not be dissolved and existed as inclusion bodies in the cells. Therefore, to avoid the problem of solubilization and renaturation that are needed to convert protein product into biologically active form, utilization of eukaryotic expression system would be more appropriate as the product should be expressed in its native form. *Pichia pastoris*, an industrial methylotrophic yeast that is capable of metabolizing methanol as its sole carbon source, was chosen as an expression system for the putative molt-inhibiting hormone of *Penaeus monodon* (Pem-MIH) in this study.

Material and methods

Amplification of putative mature Pem-MIH cDNA

A cDNA encoding the mature Pem-MIH was obtained by amplification of the first stranded cDNA from eyestalk using gene specific primers MIH-F and MIH-R1 that were designed from the 3' and 5' RACE fragments of Pem-MIH (Fig. 1). The amplification by polymerase chain reaction (PCR) was performed in a total volume of 50 µl containing 4 µl of first stranded cDNA, 0.2 µM of each primer, 0.2 µM dNTP, 1.5 mM Mg^{2+} and 2.5 units of Taq DNA polymerase (Biotool). Amplification was performed in a DNA thermal cycler (GeneAmp System 2400, PE applification Biosystems) with 35 cycles under the following condition: 95 ^{o}C for 30 seconds, 50 ^{o}C for 30 seconds and 72 ^{o}C for 1 minute. The last PCR cycle was followed by a final extension at 72 ^{o}C for 7 minutes. One microliter of the resulting PCR product was subjected to a secondary amplification in order to increase the specificity of the amplified product by using gene specific primers MIH-F and MIH-R2.1. The amplification was performed with the same condition as primary amplification.



Oligo nucleotide primers

Forward primer *EcoR*I *Sal*I Kex2 start

MIH-F : 5' CTTCGAATTCGTCGACAAAAGA*AGT*TTCATAGACGGCAC 3'

Reverse primers

MIH-R1 : 5' GAGCGAGGCCTTGCAAGTGGAAGGAG 3'

*Xba*I stop

MIH-R2.1 : 5' GCGTTCTAGA*TCA*CTGACCGGCGTTCAGGATG 3'

Fig. 1 Nucleotide sequence of putative Pem-MIH cDNA derived from the sequence of 5' and 3' RACE fragments (previous work). The 15 bp overlapping sequence is black shaded. The 315 bp open reading frame is depicted by capital letters. The deduced amino acids are written in one-letter symbols. The mature peptide starts from block arrow. Black arrows represent primers.

Construction of expression vector

A cDNA encoding the mature Pem-MIH fragment was cloned into pPICZαA expression vector by inserting downstream of α-factor secretion signal. In the resulting construct, pPICZα A/MIH (Fig.2), the Pem-MIH cDNA was placed under the control of *AOX1* promoter and the expressed product was expected to be secreted into the culture medium by the α-factor secretion signal that contains no Glu-Ala repeats. The construct, pPICZαA/MIH was then transformed into *E. coli* strain DH5α and the recombinant clones were selected on LB agar plate supplemented with 25 μg/ml Zeocin™. The transformants clones were subjected to PCR

screening and the DNA sequence of the positive clones were determined by the method of ABI PRISM™ using ABI PRISM Model 377 Dye terminator cycle Automate DNA sequencer (PE Applied Biosystems).

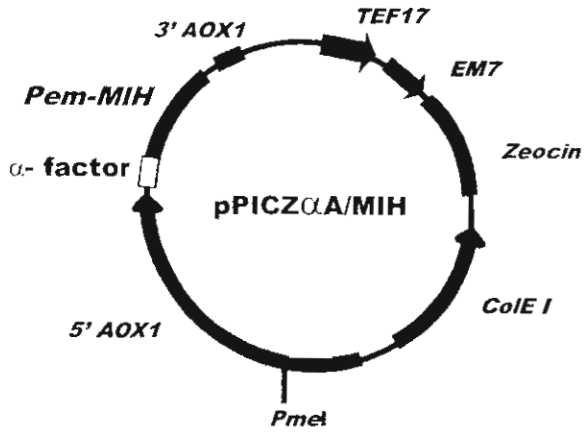


Fig 2. A physical map of pPICZαA/MIH. The recombinant vector contains Pem-MIH cDNA that fused in-frame with the α-factor secretion signal without Glu-Ala repeats. The fusion protein is under the control of *5' AOX1* promoter. *TEF1* and *EM7* promoter drive expression of the Zeocin™ resistance gene in *Pichia* and *E. coli*, respectively. *ColE1* allows replication and maintenance of the vector in *E. coli*.

Transformation of Pichia pastoris by electroporation

The recombinant vector, pPICZαA/MIH, were linerized by *Pme*I within the 5' *AOX1* region. About 500 ng of linearized vectors were precipitated, resuspended in 5 μl sterile distill water and subsequently incubated with *P. pastoris* competent cells. The mixture was transfer to an ice-cold 0.2 cm electrocuvette and pulsed by using Bio-Rad Gene Pulser with the condition: 1.5 kV, 2.5 μF and 200 Ω. The transformed reaction was added with 1 ml of 1 M sorbitol and incubated at 30 °C without shaking for 1 h. Then, 1 ml of YEPD was added and the incubation was continued at 30 °C with shaking for 2 h. The *P. pastoris* recombinant were selected on YEPD plate supplemented with 100 μg/ml Zeocin™ and subsequently analyzed for the integration of pPICZαA/MIH into *P. pastoris* genome by using PCR amplification with *5'AOX1* and *3'AOX1* primers.

Small scale expression of recombinants Pem-MIH peptide in P. pastoris.

A single colony of recombinant *P. pastoris* was inoculated in 2 ml YEPD supplemented with 100 μg/ml Zeocin™ at 30 °C with shaking for 72 h. The culture was transferred to 5 ml BMGY medium, starting at OD_{600} = 0.2, and grown at the same condition until OD_{600} reach 5 - 6. In the induction step, the cell pellet was harvested by centrifuge at 5,000 rpm for 5 minutes at room temperature, then resuspended in 1 ml BMMY medium. Absolute methanol was added

to a final concentration of 3% (v/v) every 24 h to maintain the induction for 3 days. To monitor Pem-MIH expression and secretion from the cell, the culture supernatant was analyzed on 16.5% Tricine SDS-PAGE.

Results

Amplification of putative mature Pem-MIH cDNA

By using gene specific primers to amplify the cDNA encoding the mature Pem-MIH, a single band of amplification product was obtained (Fig. 3) and subsequently cloned into pPICZαA expression vector. The nucleotide sequence analysis revealed a 231 bp open reading frame encoding 77 residues of Pem-MIH. The deduced amino acid sequence of Pem-MIH was compared with MIH, GIH and CHH peptide from other crustaceans (Fig. 4). Pem-MIH showed the highest similarity in its structure to that of the processed active MIH of *P. japonicus*, Pej-MIH (Ohira et al., 1997)

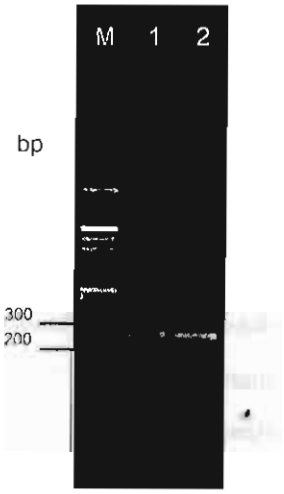


Fig. 3 The PCR products amplified using gene specific primers.
M: 100 bp DNA ladder markers.
Lane 1: Primary amplification using MIH-F and MIH-R1 primers
Lane 2: Secondary amplification using MIH-F and MIH-R2.1

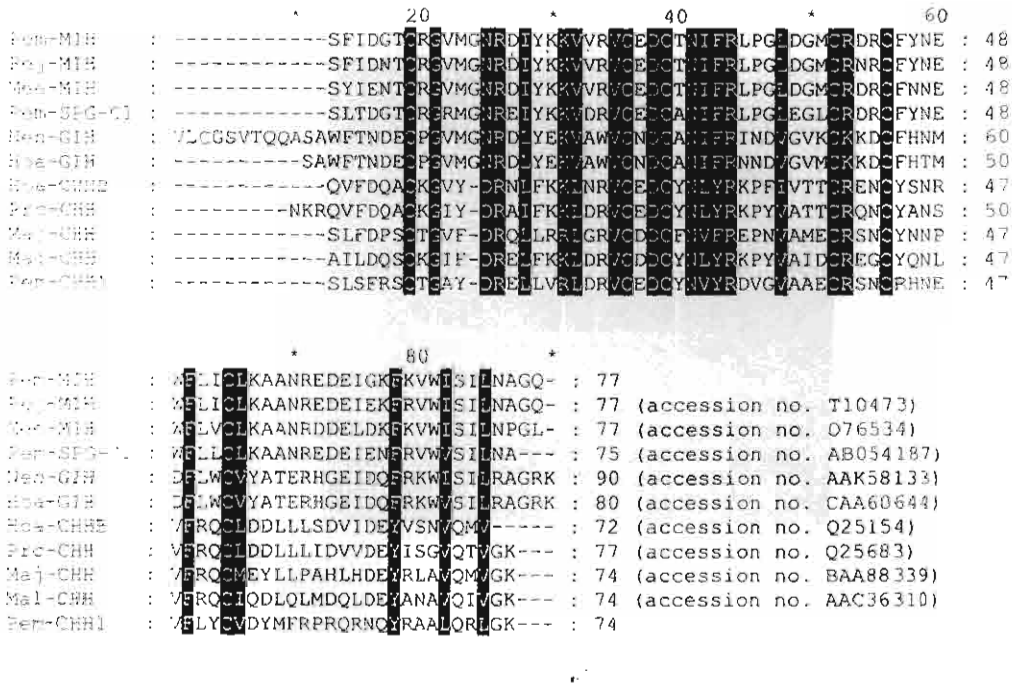


Fig. 4 Comparison of the amino acid sequence of Pem-MIH peptide with those of *P. japonicus* MIH (Pej-MIH, Ohira et al., 1997), *M. enesis* MIH (Mee-MIH, Gu et al., 2000), *P. monodon* sinus gland peptide (Pem-SPG-C1, Krugkasem et al., 2002), *Nephrops norvegicus* GIH (Nen-GIH, Edomi et al., 2002), *H. americanus* GIH (Hoa-GIH, De Kleijn et al., 1994), H. americanus CHHB (Hoa-CHHB, De Kleijn et al., 1995), *Procambarus clarkii* CHH (Prc-CHH, Yasuda-Kamatani et al., 1999), *Masupenaeus japonicus* CHH (Mej-CHH, Ohira et al., 1999), *Macrobrachium lanchesteri* CHH (Mal-CHH, Ju et al., 1998) and *P. monodon* CHH1 (Pem-CHH1, Treerattrkool, 2001). The amino acid residues that are identical to Pem-MIH peptide are highlighted.

Determination of Pem-MIH cDNA integration into P.pastoris genome.

The recombinant vector pPICZαA/MIH was linearized with *Pme*I before transformed into *P. pastoris*. Digestion with *Pme*I provides the integration region for Pem-MIH cDNA fragment to integrate into *P. pastoris* genome. *P. pastoris* transformants were firstly screen for Zeocin™-resistance using YEPD supplemented with 100 μg/ml Zeocin™ plate. Genomic DNA of Zeocin™ transformants were extracted and further identified for integration by PCR analysis using *5' AOX1* and *3' AOX1* primers. PCR screening of ten *P. pastoris* transformants that were transformed with pPCZαA/MIH showed PCR products with the sizes of 754 bp, while a band of 589 bp was observed from transformants containing only pPICZαA expression vector (Fig. 5). These results suggest that the linearized vectors containing Pem-MIH fragment was integrated into the *P. pastoris* genome in all ten clones.

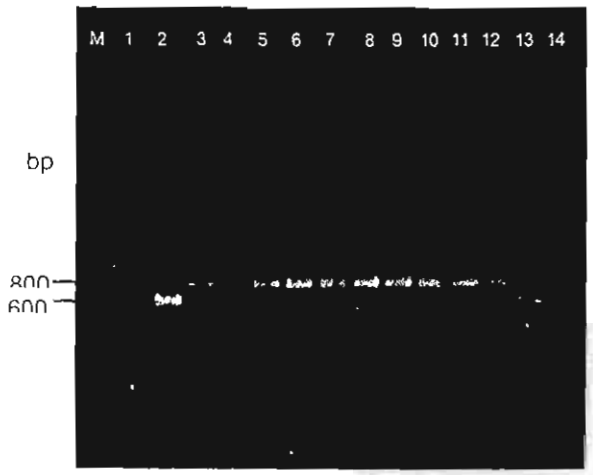


Fig. 5 PCR screening for integration of pPICZ αA fecombinant plasmids into the genome of *P. pastoris* transformants.

M: 100 bp DNA ladder markers

Lane 1: PCR product of *P. pastoris* genome

Lane 2: PCR product of transformants containing integrated pPICZ αA vector.

Lane 3 –12: PCR product of transformants containing integrate d pPICZαA/MIH plasmids.

Lane 13: PCR product of pPICZ αA plasmid.

Lane 14: negative control in the absence of DNA template.

Small scale expression of recombinant Pem-MIH

The positive clones that contains Pem-MIH fragment integration were chosen for small scale expression in order to select for the clones that give highest level of expression. These clones were grown in BMGY medium until OD_{600} reach 5-6. To induce the expression, cells were harvested and resuspend in BM containing 1% (v/v) of methanol. The methanol was subsequently added to final concentration of 3 % (v/v) every 24 h. for 3 days. The secreted Pem-MIH expression was detected by 16.5 % Tricine SDS-PAGE as a band at the expected size about 8.4 kDa (Fig. 6). The three highest level expression clones (clone 22, 25 and 28 in lane 7, 9and 12 respectively) secretedabout 5 mg/l of Pem-MIH into culture medium.

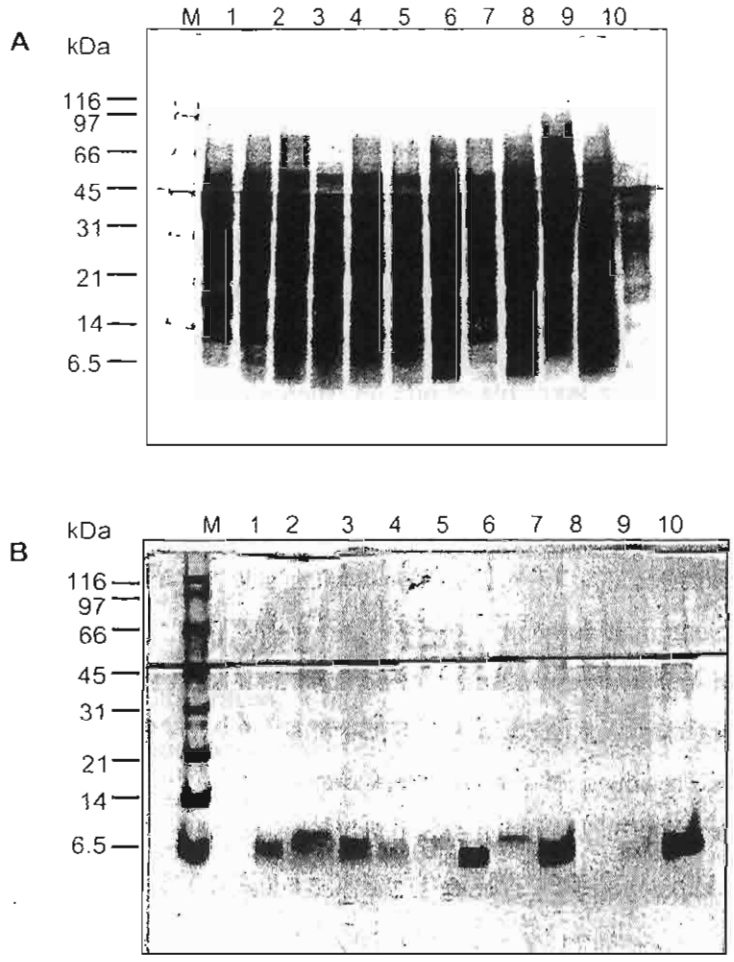


Fig. 6 Tricine SDS-PAGE protein profile of the *P. pastoris* transformants containing pPICZ αA/MIH integration. Panel A and B show total lysate OD_{600}= 0.2 and 100 µl culture medium of *P. pastoris* recombinant after methanol induction, respectively.

M : Molecular weight marker

Lane 1: pPICZαA *P. pastoris* transformants

Lane 2: pPICZαA/CHH *P. pastoris* transformants

Lane 3 -12: pPICZαA/MIH *P. pastoris* transformants.

Discussion

The open reading frame of Pem-MIH peptide from *P. monodon* was successfully cloned. The deduced amino acid sequence reveals a putative mature peptide comprising 77 amino acid residues and shares the highest degree of identity with process peptide of MIH of *P. japonicus* (94%). Significant degrees of identity were observed between Pem-MIH and MIH

of *Metapenaeus ensis* (87%), including recently report of *P. monodon* sinus gland peptide (Pem-SGP-C1, 80%). On the contrary, lower degrees of identity were found between Pem-MIH and GIH (47%) and CHH-B (32%) of the american lobster, *Homarus americanus*. Pem-MIH also showed low level of identity (29%) in the amino acid sequences to CHH1 of *P. monodon* (Treerattrakool, 2001)

Chan et al., (1990) has shown that the purified peptide from the sinus gland of *H. americanus* had both MIH and CHH activity. Moreover, Cam-MIH and Pej-SGP-VI were also demonstrated to posses both functions (Webster, 1991 and Yang et al., 1996). This is not surprising as the overlapping activities could be due to structural similarity among the peptides in this family. The high degree of identity between these peptides not only depends on species, but also correlates with a highly conserved function of these hormones. In contrast, the MIH peptide of *P. bouvieri* that share 90% identity in the amino acid sequence with its own CHH does not present any hyperglycemic activity (Aguilar-Gaytan et al., 1997). However, to conclude that Pem-MIH is a molt-inhibiting hormone, physiological study is necessary to clarify whether this hormone can prolong molt duration.

The recombinant Pem-MIH was expressed in *P. pastoris* as a secreted protein with higher yield than the Mee-MIH that was expressed in *E. coli* (Gu et al., 2001). Although the N - terminus of Mee-MIH contains 47 residues of the additional amino acids that could have effect to its biological activity, Mee-MIH significantly extended molt duration of juvenile *M. ensis*. For the recombinant protein expressed in *P. pastoris*, the additional N - terminus peptides of the α-factor secretion signal is removed by KEX2 protease. The cleavage at KEX2 cleavage site could be facilitated by the present of the juxtapose downstream Glu-Ala repeats (Romanos et al.,1995). However, the recombinant Pem-CHH1 expressed in *P. pastoris* has been demonstrated to be completely processed by KEX2 protease without the Glu-Ala repeats and also retained biological activity (Treerattrakol, 2001). This result indicates that the cleavage between the α-factor signal sequence and Pem-CHH1 at the KEX2 site did not necessarily require the Glu-Ala repeats. However, the N-terminal region of the secreted Pem-MIH peptide need to be determined in order to ensure that the α-factor secretion signal was completely cleaved by KEX2 protease in the absent of Glu-Ala repeat. Since the isolation of MIH from eyestalks using HPLC (High - performance liquid chromatography) and other techniques have their limitation, recombinant DNA technology may provide an alternative approach to obtain a large quantity of pure and biological active protein to study the structural and function relationships of MIH including determination of a precise mechanism of molt inhibition.

Acknowledgements

We thank Ms Supattra Treerattrakool (Prawn Molecular Biology laboratory, Institute of Molecular Biology and Genetics, Mahidol University) for suggestion in *Pichia pastoris* expression. This study was supported by the Thailand Research Fund (TRF).

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บทคัดย่อ

ABSTRACTS

การประชุมวิชาการ
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FACULTY OF SCIENCE, KHON KAEN UNIVERSITY

การศึกษาลักษณะและหน้าที่ของ PUTATIVE MOLT-INHIBITING HORMONE ในกุ้งกุลาดำ

MOLECULAR CHARACTERIZATION AND BIOLOGICAL ASSAY OF A PUTATIVE MOLT-INHIBITING HORMONE OF PENAEUS MONODON

สุพรรษา ยอดเมือง, อภินันท์ อุดมกิจและ สกล พันธุ์ยิ้ม.

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บทคัดย่อ: การเจริญเติบโตของสัตว์จำพวก ครัสเตเซียน ต้องอาศัยกระบวนการทางสรีรวิทยาที่เรียกว่า กระบวนการลอกคราบ ซึ่งถูกกระตุ้นโดย ecdysteroid ที่สังเคราะห์มาจาก Y-organ การสร้าง ecdysteroid จะถูกควบคุมโดย molt-inhibiting hormone (MIH) ที่ผลิตโดยกลุ่มประสาทบริเวณก้านตาที่เรียกว่า X-organ sinus gland complex (XOSG) ชิ้น cDNA ของ mature MIH ในกุ้งกุลาดำ (*Penaeus monodon)* ถูกโคลนและนำไปแสดงออกในยีสต์ *Pichia pastoris* โปรตีนลูกผสม Pem-MIH ที่ผลิตได้ถูกนำไปทำให้บริสุทธิ์โดยวิธี size exclusion chromatography และเมื่อทดสอบหน้าที่ของ Pem-MIH ในกุ้งกุลาดำ พบว่ากุ้งที่ได้รับ Pem-MIH จะใช้เวลาในการเกิดการลอกคราบครั้งต่อไปนานกว่ากุ้งในกลุ่มควบคุมอย่างมีนัยสำคัญ ดังนั้นจึงกล่าวได้ว่าโปรตีนลูกผสมที่ได้จากการทดลองนี้คือ molt-inhibiting hormone

Abstract: Growth in Crustacean occurs only when the exoskeleton is removed by a physiological process called molting. Molting is triggered by ecdysteriods that are synthesized and secreted from the Y-organ. The synthesis of ecdysteroids is negatively regulated by molt-inhibiting hormone produced by X-organ sinus gland complex (XOSG) in the eyestalk. A cDNA encoding the mature region of molt-inhibiting hormone of *Penaeus monodon* (Pem-MIH) was cloned. Recombinant protein of the expected size for MIH was expressed form Pem-MIH cDNA in the yeast *Pichia pastoris*. The recombinant Pem-MIH was purified by size exclusion chromatography. Biological activity assay revealed that recombinant Pem-MIH could significantly prolong the molt duration of *P. monodon* suggesting it function as a molt-inhibiting hormone.
Key word: MIH, molt duration, Black tiger shrimp

Methodology: 1. Cloning of a cDNA encoding the mature molt-inhibiting hormone of *Penaeus monodon* (Pem-MIH). Total RNA from optic ganglia was used as a template to synthesize the first strand cDNA by using an oligo-dT primer and Superscript™ II reverse transcriptase (Gibco-BRL). The resulting first strand cDNA was subjected to PCR amplification by gene specific primers, MIH-F and MIH-R2.1. The PCR product was subsequently clone and its nucleotide sequences determined. 2. Expression of recombinant Pem-MIH peptide. The cDNA encoding Pem-MIH was ligated to pPICZαA expression vector downstream of α-factor secretion signal. The resulting plasmid, *αMIH-EX was Pme* I endonuclease before transformed into a methylotrophic yeast, *Pichia pastoris*. The recombinant clones were determined for the integration of *aMIH-EX* into *P. pastoris* genome by using PCR amplification with *5'AOX1* and *3'AOX1* primers. A single colony of recombinant *P. pastoris* was grown in BMGY Medium at 30 °C with

shaking until OD_{600} reach 5-6. The cell pellets were then harvested and resuspended in to1/5 volume in BMMY medium. The expression was induced by the addition of absolute methanol to a final concentration of 3% (v/v) every 24 h for 5 days. The secreted protein in the culture supernatant was determined by 16.5% SDS-PAGE and the recombinant Pem-MIH was purified on Superdex 75 column (Amersham pharmacia biotech). 3. In vivo bioassay of Pem-MIH. Individual live black tiger shrimps (13.5-14 gm each) were maintained in 10 ppt aerated artificial seawater in separate compartments. Shrimps were allowed to molt once and leaved for 3 days before injected with 5 μg purified Pem-MIH. Shrimps in control group were received an equal volume of PBS injection. Molt duration was determined by the time that the shrimps tool for completing the next round of molting.

Results, Discussion and conclusion: The total RNA from of a shrimp was used as a template for cDNA cloning in order to exclude polymorphism among individuals. A PCR product of the expected size of 231 bp encoding 77 amino acids was obtained. The deduced amino acid sequences from the cDNA share the highest degree of identity (94%) with the processed peptide of MIH of *P. japonicus*. Significant degree of identity was observed between Pem-MIH and MIH of *Metapenaeus ensis* (87%). Pem-MIH also shares 29% identity to crustacean hyperglycemic hormone1 of *P. Monodon* (Pem-CHH1) previously shown to have the ability to elevate haemolymph glucose level. A peptide purified from the sinus gland of the lobster, *Homarus americanus* had been shown to processes both MIH and CHH activity. It is not surprising that overlapping activities were due to closely related structures among peptides in this family. Typically six cycteine residues are conserved an identical positions in all of these hormones. Determination of the molting period showed that molting took place within a duration of 11.8 ± 1.48 days for the *P. monodon* infected with PBS. In the biological assay for MIH activity of the recombinant Pem-MIH, the length of molt duration was significantly extended to 16.29 ± 1.98 days for shrimps injected with purified recombinant Pem-MIH. There was no significant difference in the molt cycle duration for shrimp injected with crude eyestalk extract compared to shrimp injected with purified Pem-MIH. These results suggested that Pem-MIH function to prolong the molt duration, and thus are considered as a molt-inhibiting hormone of *P. monodon*.

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การยับยั้งการทำงานของฮอร์โมนเพิ่มระดับน้ำตาล (CHH) ในกุ้งกุลาดำ โดยแอนติบอดี้ต่อโปรตีนลูกผสม Pem-CHH 1

THE INHIBITORY EFFECT OF ANTI-rPem-CHH 1 ANTIBODY ON THE ACTIVITY OF CRUSTACEAN HYPERGLYCEMIC HORMONE (CHH) IN Penaeus monodon.

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บทคัดย่อ : Crustacean hyperglycemic hormone (CHH) เป็นเปปไทด์ฮอร์โมนที่สร้างจากเซลล์ประสาทหลั่งสารของ X-organ ในก้านตาของสัตว์จำพวกครัสเตเซียน CHH ทำหน้าที่ควบคุมระดับน้ำตาลในเลือดและเกี่ยวข้องกับการพัฒนาของไข่ในระบบสืบพันธุ์ ได้มีการศึกษาเกี่ยวกับการโคลนนิ่ง และ หน้าที่ของ CHH ในกุ้งกุลาดำ (Pem-CHH) ในงานวิจัยก่อนหน้านี้ ในการศึกษานี้ได้ทำการสร้างโปรตีนลูกผสม CHH 1 ของกุ้งกุลาดำ (rPem-CHH 1) ในระบบของแบคทีเรีย และนำมาใช้ในการสร้างแอนติบอดี้ในหนู แอนติบอดี้ที่สร้างได้มีความจำเพาะต่อ rPem-CHH 1 จากการศึกษา พบว่าแอนติบอดี้ต่อ rPem-CHH 1 ยับยั้งความสามารถในการเพิ่มระดับน้ำตาลในเลือด ของสารสกัดจากก้านตาของกุ้งกุลาดำได้ประมาณ 50 % นอกจากนี้ แอนติบอดี้ต่อ rPem-CHH 1 ยังสามารถยับยั้งการทำงานของฮอร์โมน CHH ในกุ้งกุลาดำ ได้ประมาณ 15-20%.

Abstract: Crustacean hyperglycemic hormone (CHH), produced from the X-organ sinus gland complex in the eyestalk of crustaceans, is responsible for glucose level in the haemolymph and also plays role in ovarian development. The cloning and characterization of recombinant CHH 1 of *Penaeus monodon* (rPem-CHH 1) has been previously reported. In this study, large quantity of rPem-CHH1 was obtained by expression in bacterial expression system, and was used for antibody production in mice. The antibody obtained showed specificity to rPem-CHH1. This anti-rPem-CHH 1 antibody reduced the ability to elevate the haemolymph glucose level of the eyestalk crude extract to about 50 %. Moreover, the antibody could also inhibit about 15-20% of the activity of the natural CHH in *P. monodon*.

Methodology: 1 Expression and Purification of rPem-CHH 1. Using *E. coli* expression system, the rPem-CHH 1 protein was expressed after induction with 0.4 mM IPTG for 4 h. The inclusion form of rPem-CHH 1 was solubilized in 8M Urea/PBS (pH 7.4). The soluble protein was subsequently purified by size-exclusion chromatography. 2 Antibody production against rPem-CHH 1. The purified rPem-CHH 1 was transferred to a buffer containing 0.2% (w/v) SDS in PBS, pH 7.4 by Centriplus YM-3 column (Millipore, USA). The female mice (BALB/C) were immunized with the mixture between 100 μg of purified rPem-CHH 1 and complete Freud's adjuvant (Sigma). Subsequently the antibody production was boosted with the mixture between 100 μg of purified rPem-CHH 1 and incomplete Freud's adjuvant in the second and third injections. The antiserum of whole blood from the heart was determined for its sensitivity and specificity, and used in biological assay. 3 Biological assay for inhibitory effect of anti-rPem-CHH 1 antibody on CHH activity. 3.1 One pair-equivalent of eyestalk crude extract was pre-incubated with anti-rPem-CHH 1 antibody in a final dilution of 1:500 at 4°C for 2 h. This solution was injected into ten individual starved eyestalk-ablated *P. monodon* through the arthodial membrane of the second walking leg. For the negative and positive control groups, each individual shrimp was injected with 100 μl of PBS and one pair-equivalent of eyestalk crude extract, respectively. 3.2 Anti-rPem-CHH 1 antibody (1:500) was injected into twenty individual normal shrimps while the control group was injected with 100 μl of PBS. The glucose level in the haemolymph of individual shrimp in 3.1 and 3.2 were determined at 0, 0.5,1, 1.5 h after injection using a glucose dignostic kit (Sigma).

Results, Discussion and Conclusion: The rPem-CHH 1 protein was expressed as inclusion bodies. Generally, inclusion bodies can be solubilized in high concentration of denaturant such as urea or guanidine-HCl. In this study, the inclusion bodies of rPem-CHH 1 were solubilized in 8M urea/PBS (pH 7.4). The soluble fraction was purified by size-exclusion FPLC chromatography. This purification could remove all contaminated protein from rPem-CHH 1. The urea in the buffer was removed by replacing with 0.2% (w/v) SDS in PBS, pH 7.4 in Centriplus YM- 3 column. The low concentration of SDS would increase solubility of rPem-CHH 1. The final yield of purified rPem-CHH 1 was 12 mg/L of the culture medium as determined by Bradford's method using the Bio-Rad Protein Assay kit. Mouse antiserum was raised against purified rPem-CHH1. The sentivity of anti-rPem-CHH 1 antibody was determined by dot blot analysis. Five nanograms of purified rPem-CHH could be detected by a 1:20,000 dilution of the antibody. Anti-rPem-CHH 1 antibody specifically recognized CHH. The ability to elevate the haemolymph glucose level of the eyestalk crude extract decreased dramatically (50%) after pre-incubated with the antibody suggesting that the binding of antibody to CHH in the eyestalk extract can inhibit its hyperglycemic activity. Moreover, *P. monodon* injected with anti-rPem-CHH 1 antibody exhibited about 15-20 % decrease in hyperglycemic activity suggesting that anti-rPem-CHH 1 antibody could also inhibit the activity of the natural CHH in *P. monodon* at some extent.

Reference: (1) Udomkit, A., Chooluck, S., Sonthayanon, B., and Panyim, S. 2000. J. Exp. Mar. Biol. Eco.224, 145-156.

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Keywords: Antibody, Crustacean hyperglycemic hormone (CHH), *Penaeus monodon*.

THE INHIBITORY EFFECT OF ANTI-rPem-CHH I ANTIBODY ON THE ACTIVITY OF CRUSTACEAN HYPERGLYCEMIC HORMONE (CHH) IN *Penaeus monodon*.

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Abstract

Crustacean hyperglycemic hormone (CHH), produced from the X-organ sinus gland complex in the eyestalk of crustaceans, is responsible for glucose level in the haemolymph and also plays role in ovarian development. The cloning and characterization of recombinant CHH 1 of *Penaeus monodon* (rPem-CHH 1) has been previously reported. In this study, large quantity of rPem-CHH1 was obtained by expression in bacterial expression system, and was used for antibody production in mice. The antibody obtained showed specificity to rPem-CHH1. This anti-rPem-CHH 1 antibody reduced the ability to elevate the haemolymph glucose level of the eyestalk crude extract to about 50 %. Moreover, the antibody could also inhibit about 15-20% of the activity of the natural CHH in *P. monodon*.

Introduction

A novel peptide family in crustaceans comprising the crustacean hyperglycemic hormone (CHH), molt-inhibiting hormone (MIH) and gonad-inhibiting hormone (GIH) is produced in and released from the the major neuroendocrine control center in the eyestalk called X-organ sinus gland (XOSG) complex. CHH is the most abundant eyestalk hormone, and the biological assay for its activity is well-established (1,2). In addition to its principle function in blood glucose regulation (3), it is also involved in ovarian development (4). In *Penaeus monodon*, a cDNA encoding CHH has been identified and characterized (5).The expressed recombinant protein from this cDNA showed the ability to elevate glucose level in the haemolymph of eyestalk-ablated *P. monodon* (6). In this work, large quantity of recombinant Pem-CHH1 (rPem-CHH1) was obtained and used for antibody production in mice. The inhibitory effect of anti-CHH 1 antibody on CHH activity was investigated.

Methodology

1. Expression and Purification of rPem-CHH 1.

Using *E. coli* expression system, the rPem-CHH 1 protein was expressed after induction with 0.1 mM IPTG for 4 h. The inclusion form of rPem-CHH 1 was solubilized in 8M Urea/PBS (pH 7.4). The soluble protein was subsequently purified by size-exclusion chromatography.

2. Antibody production against rPem-CHH 1.

The purified rPem-CHH 1 was transferred to a buffer containing 0.2% (w/v) SDS in PBS, pH 7.4 by Centriplus YM-3 column (Millipore, USA). The female mice (BALB/C) were immunized with the mixture between 100 µg of purified rPem-CHH 1 and complete Freud's adjuvant (Stegrad). Subsequently the antibody production was boosted with the mixture between 100 µg of purified rPem-CHH 1 and incomplete Freud's adjuvant in the second and third injections. The antiserum of whole blood from the heart was determined for its sensitivity and specificity, and tested in biological assay.

3. Biological assay for inhibitory effect of anti-rPem-CHH 1 antibody on CHH activity.

3.1 One pair-equivalent of eyestalk crude extract was pre-incubated with anti-rPem-CHH 1 antibody in a final dilution of 1:500 at 4°C for 2 h. This solution was injected into two individual starved eyestalk-ablated *P. monodon* through the arthrodial membrane of the second walking leg. For the negative and positive control groups, each individual shrimp was injected with 100 µl of PBS and one pair-equivalent of eyestalk crude extract, respectively.

3.2 Anti-rPem-CHH 1 antibody (1:500) was injected into twenty individual normal shrimps while the control group was injected with 100 µl of PBS.

The glucose level in the haemolymph of individual shrimp in 3.1 and 3.2 were determined at 0, 0.5, 1, 2, 4, 6 h after injection using a glucose diagnostic kit (Sigma).

Results and Discussion

The rPem-CHH 1 protein was expressed as inclusion bodies. Generally, inclusion bodies can be solubilized in high concentration of denaturant such as urea or guanidine-HCl. In this study, the inclusion bodies of rPem-CHH 1 were solubilized in 8M urea/PBS (pH 7.4). The soluble fraction was purified by size-exclusion FPLC chromatography. This purification could remove all contaminated protein from rPem-CHH 1 (Figure 1). The urea in the buffer was removed by replacing with 0.2% (w/v) SDS in PBS, pH 7.4 in Centriplus YM- 3 column. The low concentration of SDS would increase solubility of rPem-CHH 1. The final yield of purified rPem-CHH 1 was 12 mg/L of the culture medium as determined by Bradford's method using the Bio-Rad Protein Assay kit. Mouse antiserum was raised against purified rPem-CHH1. The sensitivity of anti-rPem-CHH 1 antibody was determined by dot blot analysis. Five nanograms of purified rPem-CHH could be detected by a 1:20,000 dilution of the antibody (Figure 2). Anti-rPem-CHH 1 antibody specifically recognized CHH (Figure 3). The ability to elevate the haemolymph glucose level of the eyestalk crude extract decreased dramatically (50%) after pre-incubated with the antibody suggesting that the binding of antibody to CHH in the eyestalk extract can inhibit its hyperglycemic activity (Table 1). Moreover, *P. monodon* injected with anti-rPem-CHH 1 antibody exhibited about 15-20 % decrease in hyperglycemic activity (Table 2) suggesting that anti-rPem-CHH 1 antibody could also inhibit the activity of the natural CHH in *P. monodon* at some extent.

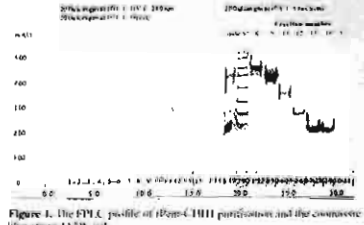

Figure 1. The FPLC profile of rPem-CHH1 purification and the coomassie blue-stained SDS gel.

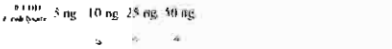

Figure2. Sensitivity of anti-CHH1 antibody by dot blot analysis.

Sample	Glucose concentration (mg/dl)	Inhibitory effect (%)
PBS	3.50 ± 1.2	

Table 1. The haemolymph glucose level at 1 h after injected with eyestalk crude extract pre-incubated with antibody.

Sample	Relative to haemolymph glucose	Inhibitory effect (%)
PBS	1.00	

Table 2. The haemolymph glucose level at 0.5 h after injected with anti-CHH1 antibody.

Figure3. Specificity of anti-CHH1 antibody.

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