Journal of Biochemistry and Molecular Biology, Vol. 35, No. 5, September 2002, pp. 476-481

JOURNAL OF
Biochemistry and Molecular Biology
© BSRK & Springer-Verlag 2002

Secretion of Pem-CMG, a Peptide in the CHH/MIH/GIH Family of *Penaeus monodon,* in *Pichia pastoris* Is Directed by Secretion Signal of the α-Mating Factor from *Saccharomyces cerevisiae*

Supattra Treerattrakool[†], Lily Eurwilaichitr[‡,*], Apinunt Udomkit[†] and Sakol Panyim[†]

[†]Institute of Molecular Biology and Genetics, Mahidol University, Salaya campus, Nakhon Pathom 73170, Thailand

[‡]BIOTEC Training Center of Genetic Engineering and Biotechnology at Institute of Molecular Biology and Genetics, Mahidol University, Salaya campus, Nakhon Pathom 73170, Thailand

Received 10 June 2002, Accepted 5 August 2002

The CHH/MIH/GIH peptide family of black tiger prawn (*Paneaus monodon*) is important in shrimp reproduction and growth enhancement. In this study, the cDNA that encodes the complete peptide that is related to the CHH/MIH/GIH family (so-called, Pem-CMG) in the eyestalk of *P. monodon* was successfully expressed in a methylotrophic yeast *Pichia pastoris* under the control of an alcohol oxidase promoter. In order to obtain the secreted Pem-CMG, a secretion signal of either the *Saccharomyces cerevisiae* α-factor or Pem-CMG was employed. The results demonstrated that αPem-CMG, either with (α2EACMG) or without (αCMG) the Glu-Ala repeats, was secreted into the medium, while Pem-CMG with its own secretion signal failed to be secreted. The total protein amount that was secreted from the transformant that contained either α2EACMG or αCMG was approximately 60 mg/l and 150 mg/l, respectively. The N-terminus of the Pem-CMG peptide of both α2EACMG and αCMG was correctly processed. This produced the mature Pem-CMG peptide.

Keywords: Glu-Ala repeats, α-Mating factor, *Penaeus monodon*, *Pichia pastoris*

Introduction

In recent years, novel peptides of the crustacean hyperglycemic hormone (CHH), molt-inhibiting hormone (MIH), and gonad-inhibiting hormone (GIH) or CHH/MIH/GIH family were demonstrated to play important roles in controlling some physiological processes in several species in crustacean, such as growth regulation and reproduction. These peptides were produced in a minute amount in the X-organ sinus gland (XOSG) complex that is located in the optic ganglia in the eyestalk of crustacean (De Kleijn and Van Herp, 1995; Charmantiner *et al.*, 1997). Thus, it would be labor-intensive to obtain sufficient peptides for purification. Recombinant technology was, therefore, employed to provide the hormone in a larger quantity for further investigation. In our previous study, cDNA from the eyestalk of the black tiger prawn or *Penaeus monodon* that related to the CHH/MIH/GIH family (so-called Pem-CMG) was cloned (Udomkit *et al.*, 2000) and expressed in *E.coli.* However, the protein product from this system was aggregated in an insoluble form. In order to avoid the problem that is associated with solubilization and renaturation that are needed to produce biologically active Pem-CMG, the Pem-CMG was instead expressed in an eukaryotic expression system.

The eukaryotic protein expression system is likely to be in its native form since the system provides the necessary processes, such as secretion and post-translational modifications (Gellissen, 2000). *Pichia pastoris* is one of the eukaryotic expression hosts that is capable of secretion of heterologous products using the α-mating factor (α-MF), the secretion signal from *Saccharomyces cerevisiae.* This signal is subsequently cleaved by an endogenous KEX2 protease. To facilitate the removal of the α-MF signal, the Glu-Ala repeats should be added immediately downstream of the Lys-Arg cleavage site (Clare *et al.*, 1991; Wagner *et al.*, 1992; Cregg *et al.*, 1993; Van Nostrand *et al.*, 1994; Raemaekers *et al.*, 1999). However, various studies demonstrated that the Glu-Ala repeats were not efficiently removed, which resulted in an extra amino acid residue extension in some of the recombinant protein (Vedvick *et al.*, 1991; Briand *et al.*, 1999; Goda *et al.*, 2000). It was also suggested by several studies that the heterologous signal sequence can be successfully used to direct the secretion of its own protein in *P. pastoris*

*To whom correspondence should be addressed.
Tel: 66-2-4419003; Fax: 66-2-4419906
E-mail: lily@biotec.or.th

(Tschopp *et al*., 1987; Ferrari *et al*., 1997; Morel and Massculie, 1997). It is, therefore, of interest to see if the secretion of the Pem-CMG peptide can be achieved using its own secretion signal or the signal of α-MF, and also to determine if the Glu-Ala repeats are necessary for the cleavage of the α-MF secretion signal.

This study, therefore, involves the construction and expression of plasmids that contain either α2EACMG, αCMG or Pem-CMG with its own signal in *P. pastoris*. SDS-PAGE and N-terminal peptide sequencing analyzed secreted proteins that are produced from each transformant type.

Materials and Methods

Construction of *P. pastoris* expression vectors containing Pem-CMG cDNA The Pem-CMG cDNA with a different type of signal sequence was amplified using primers (Table 1). The template was from the recombinant plasmid clone Pem-CMG EX2.1 (Chooluck, 1999).

Plasmid pαCMG (α-MF + Pem-CMG) was constructed by cloning the PCR fragment of mature Pem-CMG that was directly fused to the α-MF signal without Glu-Ala into the pPICZαA expression vector between the *Xho*I and *Xba*I sites.

The PCR product that harbors the mature Pem-CMG that is fused to the α-MF secretion signal with the double Glu-Ala repeats was cloned into the pPICZαA expression vector between the *Xho*I and *Xba*I sites. This produced pα2EACMG (α-MF + 2EA + Pem-CMG)

Plasmid pBCMG (Pem-CMG, including the native secretion signal) was constructed by ligating the PCR fragment of the entire Pem-CMG coding sequence with the pPICZB expression vector between the *EcoR*I and *Xba*I sites.

Automated DNA sequencing The nucleotide sequences were determined using an automated DNA sequencer (ABI PRISM™) that is based on the fluorescent-labeled terminator method (ABI PRISM™ BigDye Terminator Cycle Sequencing Kit, Smith *et al*., 1986).

The PCR reaction was performed in a total volume of 20 µl using 4 µl of terminator premix (A-, T-, C- and G- dye terminator, dATP, dCTP, dITP, dTTP, Tris-HCl, pH 9.0, $MgCl_2$, Amplitaq DNA polymerase), 300 ng of the plasmid template, and 10 pmol of the 5’AOX1 and 3’AOX1 primers. The amplification was carried out in an automated thermal cycler GeneAmp PCR system model 2400 (Perkin Elmer Cetus, USA) with the following conditions: denaturing temperature at 96ºC for 10 s, annealing temperature at 50ºC for 5 s, and extension temperature at 60ºC 4 min for 25 cycles. The dye-labeled PCR product was analyzed on the ABI PRISM Model 377 DNA sequencer. All of the constructs were sequenced twice in both directions.

Transformation of *P. pastoris* Yeast transformation was rendered using the electroporation method, as described in the manual (version F) of the *Pichia* expression kit (Invitrogen). The plasmids (pαCMG, pα2EACMG, pBCMG) were linearized with *Pme*I and used for the transformation of the *P. pastoris* strain KM71 with the following condition: 1.5 kV, 25 µF and 200 Ω. After addition with 1 M sorbitol, the cells were incubated at 30ºC without shaking for 1 h, then YEPD (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose) was added, and the incubation was continued with shaking at 30ºC for 2 h. An amount of 200 µl of yeast cells was spread on a YEPD plate that contained 100 µg/ml Zeocin™, and incubated for 2-3 days until colonies formed. The transformants were screened for integration by PCR amplification using *5’AOX*1 and *3’AOX*1 primers.

Expression and optimization of Pem-CMG A single colony (showing the highest expression level from each construct) was grown in YEPD and incubated at 30ºC with shaking at 250 rpm for 48 h. The cell culture was transferred into 100 ml of a 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]. It was grown in the same condition until the OD_{600} reached 5-6. The inoculum was then concentrated 5 times by transferring the cell pellet into 20 ml of a 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, pH 6.0 and various concentrations (0, 0.5, 1, 2, 3 and 4% (v/v)) of methanol]. One ml aliquot of the expression culture was collected at 0, 1, 2, 3, 4, 5, 6, and 7 days for a further analysis of the Pem-CMG expression.

SDS PAGE analysis The samples were mixed with a 5x loading-sample buffer (62.5 mM Tris-HCl pH 6.8, 10% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, and 0.1% (w/v) Bromphenol Blue) and heated in boiling water for 10 min before loading onto Tricine-SDS/PAGE (16.5%). The bands were visualized by staining with Coomassie Brilliant Blue R-250.

Table 1. Primers used in this study.

Expected PCR fragment	5’primer	3’primer
1. Mature Pem-CMG fused directly to the signal of α MF without Glu-Ala	5’ATGAATTCGTCGACAAAAGAAG CCTATCCTTCAGGTC 3’	5’GCTCTAGACTACTTGCCGAGCCTC TG 3’
2. Pem-CMG fused to the α-MF secretion signal with the double Glu-Ala repeats	5’ATGAATTCGTCGACAAAAGAGAG GCTGAAGCTAGCCTATCCTTCAGGTC 3’	Same as 1.
3. The entire coding sequence of Pem-CMG including the native secretion signal	5’GACTGGTTCAATTGACAAGC 3’	Same as 1.

Dot blot analysis Both the secreted and intracellular fractions were analyzed by a dot-blotting assay. The secreted product was concentrated by TCA precipitation (Bollag *et al.,* 1996); whereas, the cell from the intracellular fraction of 0.2 OD_{600} was mixed with the 5x loading sample buffer without Bromphenol blue. The protein samples were applied to a nitrocellulose membrane. Immunodetection was carried out by using a mouse anti-CMG antibody (provided by Dr. Paisan Sitigorngul, Srinakharinwirot University, Thailand) at a dilution of 1 : 5,000, and an anti-mouse IgG that was conjugated with a horseradish peroxidase-link whole antibody from a donkey. The signal was detected using Enhanced Chemiluminescence (ECL) reagents (Amersham).

N-terminal peptide sequencing N-terminal sequencing was performed using protein bands that were blotted onto a sequiblott™PVDF membrane (BIO-RAD) after separation by tricine-SDS/PAGE. The protein bands of interest were excised from the membrane and further analyzed using an ABI 492 (Applied Biosystems) automated protein sequencer.

Results and Discussion

Construction of the *P. pastoris* expression vector containing Pem-CMG To determine if the Pem-CMG can be expressed and secreted by either the α-factor (with or without Glu-Ala) or its own native signal, three different constructs (pαCMG, pα2EACMG, and pBCMG) were constructed. The PCR fragment of αCMG or α2EACMG was inserted between the *Xho*I and *Xba*I sites at the downstream of the methanol inducible alcohol oxidase (AOX1) promoter of pPICZαA (Fig. 1). This produced pαCMG or pα2EACMG. For pBCMG, the entire coding sequence of Pem-CMG cDNA (including the native secretion signal and mature Pem-CMG), was inserted between the *EcoR*I and *Xba*I sites of pPICZB (Fig. 1). The nucleotide sequences of the inserted fragments were analyzed in both directions. The result revealed that no mutation was detected in the sequences of the αCMG, α2EACMG and BCMG fragments. For αCMG and α2EACMG, the fragments were also inserted in-frame with the α-factor signal sequence of *S. cerevisiae*.

All three of the constructs were linearized with *Pme*I, which is a unique restriction site within the 5' *AOX*1 region. The *P. pastoris* transformants were then screened for Zeocin™-resistance using a rich medium (YEPD) that contained 100 µg/ml Zeocin™. Twenty transformants of each construct were chosen for further expression.

Pem-CMG was successfully expressed and secreted by the α-factor secretion signal of *S. cerevisiae* The *P. pastoris* transformants that produced the highest level of secreted Pem-CMG were chosen for further optimization. Two factors (methanol concentration and period of induction) that affect the production yield were investigated. The results (Figs. 2 A and B) suggested that the optimal condition for the expression of the αCMG transformant was induced with 3% methanol

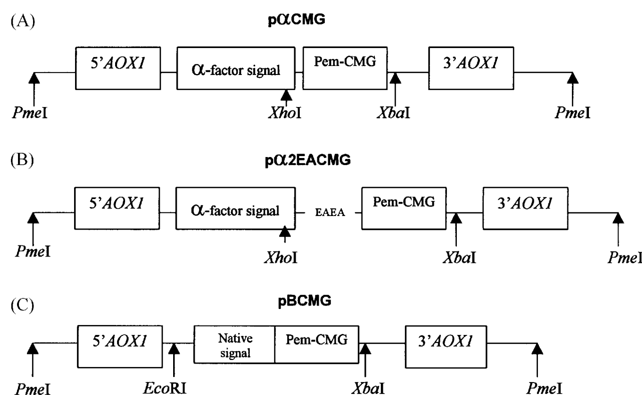

Fig. 1. Construction of the *Pichia* expression vector for secreted Pem-CMG. The linearized plasmids are consisted of the 5'*AOX1* promotor, Pem-CMG cDNA and the 3'*AOX1* sequence. (a) The cDNA encoding mature Pem-CMG peptide was cloned into the pPICZαA expression vector at *Xho*I and *Xba*I sites in-frame with the α-factor secretion signal without the spacer peptide Glu-Ala repeats. (b) The mature *Pem-CMG* cDNA was cloned into pPICZαA expression vector at *Xho*I and *Xba*I sites in-frame with the α-factor secretion signal with the spacer peptide Glu-Ala repeats. (c) The entire coding sequence of *Pem-CMG* cDNA that consists of its own native secretion signal and the mature of *Pem-CMG* cDNA was cloned into the pPICZB expression vector between *Eco*RI and *Xba*I sites.

for 3 days. For the α2EACMG transformant, the results (Fig. 3A and B) showed that the optimal condition for CMG production was to induce with 4% methanol for 3 days. The secreted level of CMG from the αCMG transformant was approximately 150 mg/l, while that of the α2EACMG transformants was approximately 60 mg/l (Table 2). N-terminal peptide sequences of recombinant Pem-CMG that was secreted from either αCMG or α2EACMG were analyzed. The result revealed that the first five amino acid residues of the secreted Pem-CMG from both constructs were Ser-Lys-Ser-Phy-Arg, which was identical to that of the mature Pem-CMG. This indicated that the MFα-1 secretion signal was completely cleaved by the KEX2 protease at the Lys-Arg sites, and that the Glu-Ala repeats of the α2EACMG construct were also completely removed by the Ste13 aminopeptidase.

Processing of the α-factor prepro-sequence in *S. cerevisiae* involved several distinct proteolytic cleavage steps. These are achieved by different enzymes. Further processing of the pro-sequence then involved the action of an endopeptidase that is encoded by the *KEX2* gene, which cleaves C-terminally to a specific Lys-Arg sequence. A dipeptidyl aminopeptidase that is encoded by the *STE13* gene removes N-terminal Glu-Ala repeats in the Golgi apparatus (Brake, 1981; Raemaekers *et al*., 1999; Cereghinno and Cregg, 2000). Although the presence of the Glu-Ala repeats enhances the activity of the Kex2 protease, subsequent processing of these repeats by the Ste13 aminopeptidase is inefficient in many cases (Brake,

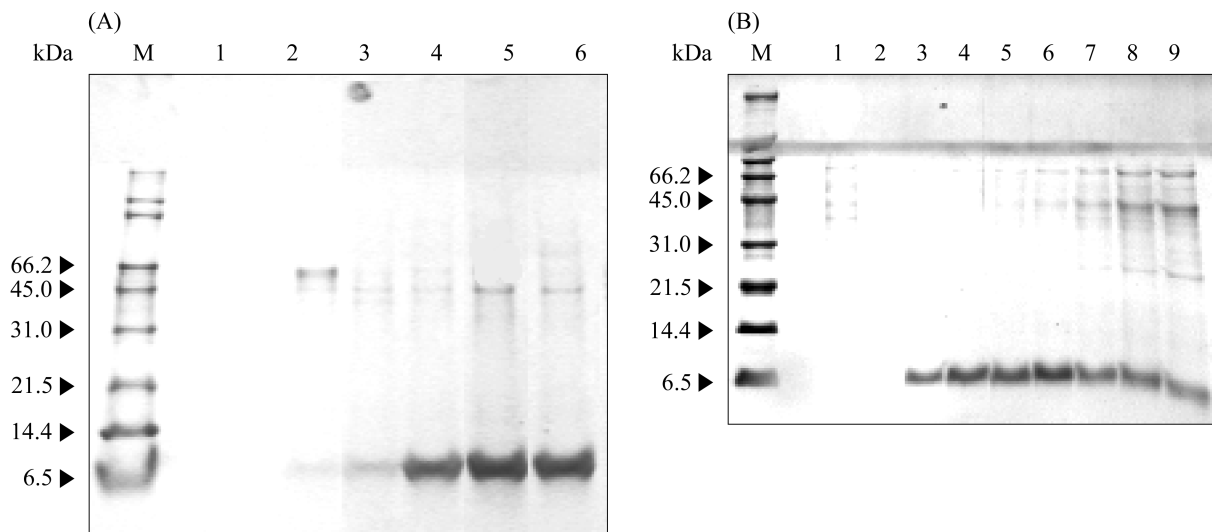

Fig. 2. Tricine-SDS analysis of the optimal expression condition from the αCMG *P. pastoris* transformant. (A) Determination of the optimal concentration of methanol. *P. pastoris* clone αCMG was induced in BM supplementing with 0, 0.5, 1, 2, 3 and 4 % (v/v) of methanol. A total volume of 100 µl of culture supernatant was collected at 4 days after induction, precipitated with TCA, and then loaded into each lane (Lanes 1-6), respectively. Lane M represents SDS-PAGE molecular weight standards, broad range protein marker (Bio-Rad, USA). (B) Determination of the optimal period of induction. αCMG was grown in BMGY and was induced in BM containing 3% (v/v) of methanol to induce the expression. A total volume of 100 µl of culture supernatant was collected at 0, 1, 2, 3, 4, 5, 6 and 7 days, precipitated with TCA, and then loaded into each lane (Lanes 2-9), respectively. Lane 1 represents 100 µl of culture supernatant of *P. pastoris* transformant containing pPICZαA vector precipitated with TCA.

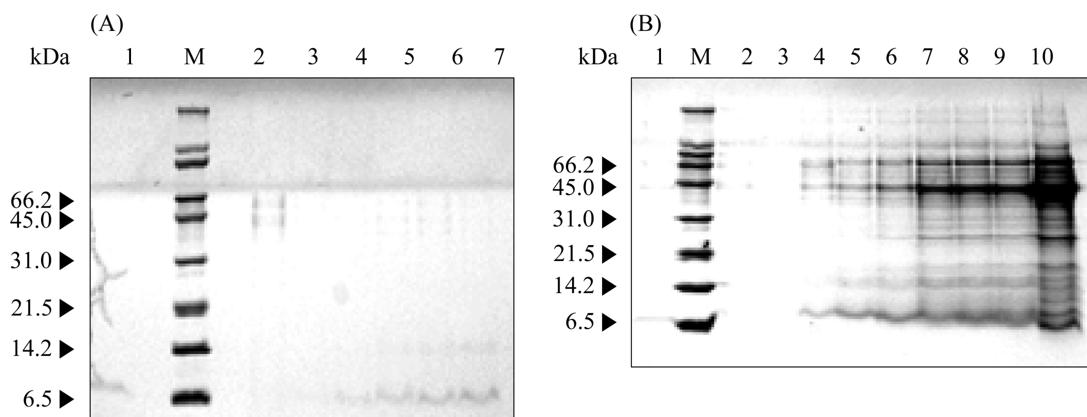

Fig. 3. Tricine-SDS analysis of the optimal expression condition from the α2EACMG *P. pastoris* transformant. (A) Determination of the optimal concentration of methanol. *P. pastoris* clone α2EACMG was grown in BMGY and was then induced in BM supplemented with 0, 0.5, 1, 2, 3 and 4% (v/v) of methanol. A total volume of 100 µl of culture supernatant was collected at 3 days after induction, precipitated with TCA, and then loaded into each lane (Lanes 2-7), respectively. Lane M represents SDS-PAGE molecular weight standards, broad range protein marker (Bio-Rad, USA). Lane 1 represents 10 µl of culture supernatant of αCMG *P. pastoris* transformant collected after induction with 3% (v/v) of methanol for 3 days. (B) Determination of the optimal period of induction. *P. pastoris* clone α2EACMG was grown in BMGY and was then induced in BM containing 4% (v/v) of methanol. A total volume of 100 µl of culture supernatant was collected at 0, 1, 2, 3, 4, 5, 6 and 7 days, and was precipitated with TCA, and then loaded into each lane (Lanes 3-10), respectively. Lane 2 represents 100 µl of culture supernatant of *P. pastoris* transformant containing pPICZαA vector precipitated with TCA.

1981; Briand *et al.*, 1999; Raemaekers *et al.*, 1999). The efficiency of both the Kex2 and Ste13 proteins can be influenced by the close proximity of the proline residues. In addition, the tertiary structure that is formed by a foreign protein may protect the cleavage site from its respective protease (Cereghinno and Cregg, 2000). The complete processing of the α-factor prepro-sequence, either with (α2EACMG) or without (αCMG) Glu-Ala repeats, indicated that the cleavage between the α-factor signal sequence and Pem-CMG at the KEX2 site did not necessarily require the

Table 2. Optimal condition for expression and yield of secreted CMG from *P. pastoris* transformants containing either αCMG, α2EACMG or BCMG

Constructs	Expressed product	Optimal period of induction (days)	% (v/v) of methanol used for induction	Total protein secretion (mg/l)
αCMG	Secretion	3	3	150
α2EACMG	Secretion	4	4	60
BCMG	Intracellular	4	3	-

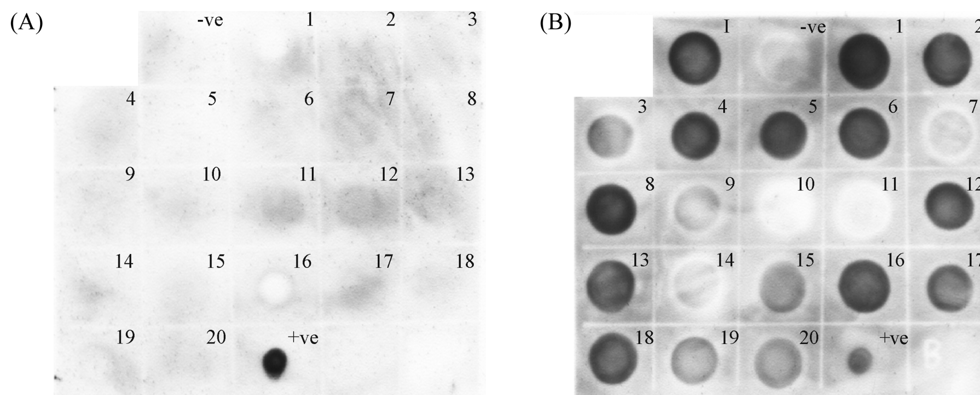

Fig. 4. Immuno-dot blotting analysis of the *P. pastoris* recombinant clones containing BCMG. Twenty BCMG *P. pastoris* transformants were grown in BMGY and were then induced in BM containing 3% (v/v) of methanol for 4 days. (A) dot blot analysis of 50 µl of culture supernatant of clones no.1-20, and -ve represents 10 µl of the culture supernatant of *P. pastoris* transformant containing pPICZB vector. (B) dot blot analysis of the intracellular fraction of 0.2 unit OD_{600} from each transformant (no. 1-20, respectively), I represents the intracellular fraction of 0.2 unit OD_{600} of αCMG *P. pastoris* transformant and −ve represents the intracellular fraction of 0.2 unit OD_{600} of *P. pastoris* transformant containing pPICZB vector. +ve in A and B represents 10 µl of the culture supernatant of *P. pastoris* transformant containing αCMG collected at 4 days after induction.

Glu-Ala repeats. In addition, the amount of the total secreted protein from the αCMG *P. pastoris* transformant was approximately twice as high as that from α2EACMG. This still needs to be investigated in order to see if the difference in the CMG expression level between the two types of transformants is due to the difference in the level of the total protein expression, or if the difference is in the efficiency to secrete the product into the culture medium.

Pem-CMG cannot be secreted by its own signal peptide in *P. pastoris* Both the secreted and intracellular fractions from all twenty clones of BCMG were determined by a dot-blotting analysis with an anti-CMG antibody. The results (Fig. 4A and B) showed that for some clones the intracellular fraction produced a positive signal; whereas, no signal was detected from the culture supernatant from any of the other clones. This indicates that Pem-CMG was successfully expressed from the BCMG transformants, but it failed to be secreted into the culture medium by its own native signal. This may be because the prepro-sequence of Pem-CMG was not recognized and processed in the yeast secretory pathway, unlike the afactor secretion signal from *S. cerevisiae*.

Although the Pem-CMG yield from the *P. pastoris* expression system may not be higher than that from the *E.coli* system (Chooluck, 1999), the product is in the soluble form and is presented as the major protein in the secreted fraction, which facilitates further protein purification. This Pem-CMG that is produced from the *P. pastoris* system is biologically active and has demonstrated hyperglycemic activity in *Penaeus monodon* (Treerattrakool, 2001).

Acknowledgments We thank Associate Professor Paisarn Sitigorngul (Srinakharinwirot University, Thailand) for providing the anti CMG-antibody. The Thailand National Center for Genetic Engineering and Biotechnology and the Thailand Research Fund (TRF) financially supported this study. The Graduated Fellowship Program, NSTDA, funded ST. SP is the TRF Senior Research Scholar.

References

Bollag, D. M., Rozycki, M. D. and Edelistein, S. J. (1996) *Concentrating Protein Solutions: Protein Methods*, Wiley-Liss, New York.

Brake, A. (1981) Secretion of heterologous protein directed by the yeast α-factor leader; in *Yeast Genetic Engineering*, Barr P. J.,

Brake, A. J. and Valenzuela, P. (eds.), pp. 269-280, Butterworth Publishers, Stoneham, Massachusetts.

Briand, L., Perez, V., Huet, J. C., Danty, E., Masson, C. and Pernollet, J. C. (1999) Optimization of the production of a honeybee odorant-binding protein by *Pichia pastoris*. *Protein Expr Purif.* **15**, 362-369.

Cereghinno, J. and Cregg, J. (2000) Heterologous protein expression in the methylotrophic yeast *Pichia pastoris. FEMS Microbiol. Rev.* **24**, 45-66.

Chooluck, S. (1999) Cloning and expression of a cDNA encoding growth-related peptide hormone of *Penaeus monodon* in *Escherichia coli* [M.Sc. Thesis in Molecular Genetics-Genetic Engineering] Mahidol University, Thailand.

Clare, J. J., Ronmanos, M. A, Rayment, F. B, Rowedder, J. E, Smith, M. A. and Payne, M. M. (1991) Production of mouse epidermal growth factor in yeast: high-level secretion using *Pichia pastoris* strains containing multiple gene copies. *Gene* **105**, 205-212.

Charmantier, G., Charmantier-Daures, M. and Van Herp, F. (1997). Hormonal regulation of growth and reproduction in crustaceans, in *Endocrinology and Reproduction, Recent Advances in Marine Biotechnology*, Vol. 1; Fingerman, M., Nagabhushanam, R. and Thomposon, M.-F (eds.), pp. 109-161, Science Publishers Inc, New Hampshire.

Cregg, J. M, Vedvick, T. S. and Raschke, W. C. (1993) Recent advances in the expression of foreign genes in *Pichia pastoris. BioTechnology* **11**, 905-910.

De Kleijn, D. P. V. and Van Herp, F. (1995) Molecular biology of neurohormone precursors in the eyestalks of crustacean. *Comp. Biochem. Physiol.* **112B**, 573-579.

Ferrari, E., Lodi, T., Sorbi, R. T., Tirindelli, R., Cavaggioni, A. and Spisni, A. (1997) Expression of a lipocalin in *Pichia pastoris*: secretion, purification and binding activity of a recombinant mouse major urinary protein. *FEBS Lett.* **401**, 73-77.

Gellissen, G. (2000) Heterologous protein production in methylotrophic yeasts. *Appl. Microbiol. Biotechnol.* **54**, 741-750.

Goda, S, Takano, K, Yamagata, Y. and Yutani, K. (2000) Effect of extra N-terminal residues on the stability and folding of human lysozyme expressed in *Pichia pastoris*. *Protein Eng.* **13**, 299-307.

Morel, N. and Massculie, J. (1997) Expression and processing of vertebrate acetylcholinesterase in the yeast *Pichia pastoris. Biochem. J.* **328**, 121-129.

Raemaekers, R. J. M., de Muro, L, Gatehouse, J. A. and Fordham-Skelton, A. P. (1999) Functional Phytohemagglutinin (PHA) and Galanthus nivalis agglutinin (GNA) expressed in the PHA-E signal peptide. *Eur. J. Biochem.* **15**, 362-369.

Smith, L. M., Sanders, J. Z., Kaiser, R. J., Hughes P., Doaa, C., Conneli, C. R., Heiner, C., Kent, S. B. H. and Hood, L. E. (1986) Fluorescence detection in automated DNA sequence analysis. *Nature* **321**, 674-679.

Treerattrakool, S. (2001) Expression of Pem-CMG of *Penaeus monodon* in *Pichia pastoris* and analysis of its biological activity [M.Sc. Thesis in Molecular Genetics-Genetic Engineering] Mahidol University, Thailand.

Tschopp, J. F., Svelow, G., Kosson, R., Craik, W. and Grinna, L. (1987) High-level secretion of glycosylated invertase in the methylotrophic yeast, *Pichia pastoris. BioTechnology* **5**, 1305-1308.

Udomkit, A., Chooluck, S., Sonthayanon, B. and Panyim, S. (2000) Molecular cloning of a cDNA encoding a member of CHH/MIH/GIH family from *Penaeus monodon* and analysis of its gene structure. *J. Exp. Mar. Biol. Ecol.* **224**, 145-156.

Van Nostrand, W. E., Schmaier, A. H., Neiditch, B. R., Siegel, R. S., Raschke, W. C., Sisodia, S. S. and Eagner, S. L. (1994) Expression purification and characterization of the kunitz type proteinase inhibitor domain of the amyloid β-protein precursor like protien-2. *Biochem. Biophys. Acta* **1209**, 165-170.

Vedvick, T., Buckholz, R. G., Engel, M., Uracan, M., Kinney, J., Provow, S., Siegel, R. S. and Thill, G. P. (1991) High-level secretion of biologically active apotinin from the yeast *P. pastoris. J. Ind. Microbiol.* **7**, 197-201.

Wagner, S. L., Siegel, R. S., Vedvivk, T. G. S., Raschke, W. C. and Van Nostrand, W. E. (1992) High-level expression, Purification and characterization of the kunitz type protease inhibitor domain of protease nexin-2/amyloid b-protein precursor. *Biochem. Biophys. Res. Commun.* **186**, 1138-1145.