ORIGINAL ARTICLE

Detection of CHH/GIH activity in fractionated extracts from the eyestalk of Banana prawn

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Abstract

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Sinus gland from *Penaeus merguiensis* De man, 1888 was extracted and proteins were fractionated on a HPLC column (µBondapack-phenyl column, 9 mm i.d., 10 µm particle size, Waters). Three major peaks (peaks 3, 5 and 6) were collected and investigated for crustacean hyperglycemic hormone (CHH) and gonad inhibiting hormone activity (GIH). The CHH activity of the peak 5 fraction was significantly higher than the control while peak 6 did not have significant CHH activity. No CHH activity was found in the peak 3 fraction. GIH activity was determined by inhibition of total protein and vitellin synthesis in ovarian tissue. An anti-vitellin antibody was prepared and used for immunoprecipitation in the GIH activity assay. Hence GIH activity was detected in peaks 5 and 6 while peak 3 had gonad stimulating hormone like activity (GSH-like activity).

Key words : anti-vitellin, crustacean hyperglycemic hormone (CHH), gonad inhibiting hormone activity (GIH), ovary, *Penaeus merguiensis*, prawn, vitellin

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การตรวจหาฤทธิ์ของฮอร์โมน CHH/GIH ในสารสกัดจากก้านตากุ้งแชบ๊วย

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การสกัดโปรตีนจากต่อมไซนัส (sinus gland) ของกุ้งแชบ๊วย (*Penaeus merguiensis* De man, 1888) และแยกโปรตีนโดยคอลัมน์ HPLC (μBondapack-phenyl column, 9 mm i.d., 10 μm particle size, Waters) สามารถแยกได้ และเก็บ 3 peaks ใหญ่ เรียกว่า peaks 3, 5 และ 6 เพื่อใช้ศึกษาฤทธิ์ฮอร์โมนที่เพิ่มระดับน้ำตาลในเลือด (crustacean hyperglycemic hormone (CHH)) และฮอร์โมนยับยั้งการเจริญของรังไข่ (gonad inhibiting hormone activity (GIH)) peak 5 พบว่ามีฤทธิ์เพิ่มระดับน้ำตาลในกระแสเลือดอย่างมีนัยสำคัญเมื่อเทียบกับสภาวะควบคุม โดยที่ peak 6 พบว่ามีฤทธิ์เพิ่มระดับน้ำตาลในกระแสเลือดอย่างไม่มีนัยสำคัญเมื่อเทียบกับสภาวะควบคุม และไม่พบฤทธิ์การเพิ่มระดับน้ำตาลในสารละลาย peak 3 สำหรับการศึกษาฤทธิ์ของฮอร์โมน GIH ได้มีการเตรียมแอนติบอดีต่อไวเทลลินเพื่อใช้ในการทำ imunnoprecipitation ในการวิเคราะห์การยับยั้งการสร้างไวเทลลิน และได้วิเคราะห์การยับยั้งการสร้างโปรตีนทั้งหมดในเนื้อเยื่อรังไข่ พบฤทธิ์ของฮอร์โมน GIH ในสารละลายของ peaks 5 และ 6 ขณะที่ peak 3 มีฤทธิ์เร่งการเจริญของgonad (gonad stimulating-like activity (GSH-like activity)

ภาควิชาชีวเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยสงขลานครินทร์ อำเภอหาดใหญ่ จังหวัดสงขลา 90112

Prawn farming is an important economic activity in Thailand. Many scientists and farmers believe that aquaculture of the banana prawn could be a good alternative to the black tiger prawn which is currently one of the major cultured species. Unfortunately, the black tiger prawn (*Penaeus monodon*) grown in aquaculture have faced many epidemic diseases and broodstock is increasingly difficult to catch. Consequently, farming technology for *Penaeus merguiensis* (De man, 1888), known as the banana prawn, is undergoing a wave of development in Thailand. However, the success of *P. merguiensis* culture requires more information in several areas. For example, the reproductive cycle of the banana prawn is not well understood and obtaining broodstock could become a problem if this species becomes a popular farming choice. Some basic knowledge of the hormones controlling the reproductive cycles as well as determining the optimum conditions for their culture is required.

Hormones released from the X-organ of crustaceans are known to regulate the optimum balance of gonadal and somatic growth processes. Gonad inhibiting hormone (GIH) released from the sinus gland is known to control gonad maturation (Charniaux-Cotton and Payen, 1985), however, its role and regulation in gonad maturation of crustaceans, especially prawns is not well understood. Knowledge of the process of gonad maturation in penaeids is still based on the model of Adiyodi and Adiyodi (1970) derived from their studies of the crab *Paratelphusa hydrodromous*. Prawns molt when the titres of molting inhibiting hormone (MIH) and gonad stimulating hormone (GSH) are low while GIH and molting hormone(s) are high. There is little evidence that proves the existence of GSH as postulated by Adiyodi and Adiyodi (1970); however, methyl farnesoate has been suggested as an active alternative gonadotropin in crustacea (Laufer *et al*., 1992). Moreover, there is a debate in the literature on how molting is controlled in crustaceans. Currently, several researchers believed that MIH and CHH (crustacean hyperglycemic hormone) control the synthesis and release of the hormone from the target tissue (Y-organ) only during intermolt. However, Chung and Webster (2003) reported that the expression of the MIH and CHH remains constant during the molt cycle and the molt control depends on the signaling machinery of the Y-organ.

GIH extracted from *Homarus americanus* appears to have a molecular weight of 9,135 Da (Soyez *et al.*, 1991), while the VIH (vitellogenesis-inhibiting hormone) of Mexican crayfish (*Procambarus bouvieri*) was 8,388 Da (Aguilar *et al.*, 1992). The amino acid and nucleic acid sequences of GIH from *Homarus americanus* (Soyez *et al.*, 1991) and *Isopod Armadillidium vulgare* (Greve *et al.*, 1999) have been determined. Several aspects of the study of GIH require a reasonable amount of pure hormone; however, there is very little GIH in the sinus glands of crustaceans. For example, the sinus gland of *Procambarus bouvieri* contains 4.5 ng of GIH (Aguilar *et al.*, 1992). Although an alternative method to obtain large amounts of GIH, such as cloning of the hormones would open the way to study many aspects of GIH, the only reported cloning of GIH has been from *Homarus americanus* and *Nephrops norvegicus* (de Kleijn *et al.*, 1994; Edomi *et al.*, 2002). CHH is the most abundant hormone in the crustacean sinus gland. CHH plays important roles in carbohydrate metabolism and osmoregulation (Charmantier-Daures *et al.*, 1994). Amino acid sequences of CHH from *Penaeus japonicus* and *Metapenaeus ensis* have been reported (Yang *et al.*, 1995; Gu *et al.*, 1998) and CHH from several species have been cloned (Gu *et al.*, 1998; Gu *et al.*, 2000; Marco *et al.*, 2003). CHH was also studied in *P. indicus*, a closely related species of *P. merguiensis*; however, the authors have not reported the amino acid sequence of CHH or detected GIH or MIH (Subramoniam *et al.*, 1998).

The present study demonstrates the isolation of GIH/CHH activities from *P. merguiensis* sinus glands. The knowledge gained from this study is crucial for future studies of these hormones.

Materials and Methods

Separation of hormones from eyestalk extract

The sinus glands were excised from *P. merguiensis* prawns harvested from the Indian Ocean and the neuropeptide hormones was extracted according to Subramonium *et al.* (1998). Briefly, after removal 1200 sinus glands were sonicated twice for 30 sec in 2 N acetic acid (1 µl/sinus gland) and incubated at 4ºC for 3 hr, then centrifuged at 18,000xg for 15 min. The supernatant was separated and the sediment was extracted repeatedly. The supernatants were pooled and separated on a µBondapak-Phenyl HPLC column, 300x3.9 mm, i.d., 10 µm particle size (Waters, USA) at a flow rate of 1ml/min. The column was eluted with a gradient of 30% of 0.1%TFA and 80% of 0.1% TFA in 60% acetonitrile. Fractions of 3 ml were collected.

CHH assay

Prawns weighing from 10-15 g were divided into 4 groups of 5. Their eyestalks were excised 2 days before injection with a fraction obtained from the HPLC column described above, except for animals in the control group which was injected with PBS (phosphate buffer saline). One hour after injection, haemolymph from each prawn was drawn and 10% sodium citrate was used as an anticoagulant. The glucose concentration of the haemolymph was determined by the glucose oxidase method (Biotech, Thailand).

Purification of vitellin and antibody preparation

Adult females of *P. merguiensis* with stage III or stage IV ovaries were selected from shrimps caught in the Indian Ocean. The ovarian stages were determined according to the gonadosomatic index (GSI), calculated as a percentage of the ovarian weight relative to body weight. Female prawns were classified into four stages: stage 1, previtellogenic (GSI, 0.2-0.5%); stage 2, early exogenous vitellogenic stage (GSI, 1.2-3.7%); stage 3, exogenous vitellogenic (GSI, 4.4-7.2%); and stage 4, late exogenous stage (GSI, 7.7-8.9%) (Tsutsui *et al.*, 2000). The ovaries were excised and weighed. Five grams of ovarian tissue were homogenized with a hand homogenizer in 20 ml phosphate buffered saline, pH 7.4 (PBS, 0.14 M NaCl, 2.68mM KCl, 10mM Na_2HPO_4, 1.76 mM KH_2PO_4) containing 0.001% PMSF (phenylmethylsulfonyl fluoride). The homogenate was centrifuged twice at 12,000xg at 4ºC for 30 min. The clear supernatant was then filtered through a

0.45 µm membrane. Ten milligrams (500 µl) of filtrate was separated by Superose 12 HR10/30 (Pharmacia, Uppsala, Sweden). The column was eluted with PBS containing 0.001% PMSF at a flow rate of 0.5 ml/min and 1 ml/fractions were collected. The protein concentration was determined by Lowry's method (Lowry *et al.*, 1951). The fractions from the column were subjected to 7% native polyacrylamide gel electrophoresis (N-PAGE) and 10% SDS-PAGE (Laemmli, 1970) for further characterisation. Proteins were detected by Coomassie Brilliant Blue G250. The native gels were also stained for lipid and carbohydrate with Sudan black B and periodic acid lipofuchsin (PAS), respectively (Clark, 1981). Purified vitellin, 500 µg in 500 µl, was mixed with an equal volume of Freund's complete adjuvant. The mixture was injected subcutaneously into a 3-month-old rabbit at 10 sites (100 µl each). A second injection was given 1 week later. A blood sample from the immunized rabbit was collected every week for 4 weeks. Serum antibody was separated and kept at -20ºC for vitellin immuno-precipitation. A crude extract of mature ovaries of *P. merguiensis* was also investigated. Ovaries were homogenized in 20 ml PBS containing 0.001% PMSF. The homogenate was centrifuged twice at 12,000x g at 4ºC for 30 min. The clear supernatant was then filtered through a 0.45 µm membrane and kept at -70ºC. The purified vitellin and the crude extract of the ovaries were separated by 7% native gel electrophoresis. The proteins on the gel were transferred to a PVDF membrane using a semi-dry blotter (Biorad, USA) at 15 V for 30 min. The membrane was incubated with 5% skim milk in PBS for 30 min followed by 1:16 anti-vitellin antibody at 37ºC for 1 hr, then washed with PBST (PBS+ 0.05% Tween 20) 3 times for 5 min. The membrane was then incubated with rabbit anti-mouse IgG-alkaline phosphatase (1:7500, Promega, USA) for 1 hr at 37ºC and washed again as previously described. The color was developed with the developing solution (10 ml of developing solution containing 0.1 M $NaHCO_3$, 1 mM $MgCl_2$, pH 9.8, 0.23 mM of BCIP (bromochloro indolyl phosphate) and 0.37 mM NBT (nitroblue tetrazolium salt) until the positive bands became sufficiently intense and the reaction was stopped by dipping the membrane in water. The membrane was then air-dried.

GIH activity assay (Quackenbush, 1992)

The fractions previously separated by HPLC were assayed for an ability to inhibit protein synthesis by measuring the incorporation of radioactive ^{14}C leucine into *P. merguiensis* stage two ovaries according to the method of Quackenbush (1992). Briefly, stage 2 ovaries of *P. merguiensis* were excised in cold normal saline and sliced into 2x3mm pieces. The sliced ovarian tissue was incubated in 1 ml sterile culture medium (1mM Hepes pH 8.2, 80 µg/ml ampicilin, 9.9g M199) which contains ^{14}C leucine (0.05 µCi/4nmol) and 100 µl of 10 eyestalks extracted. The reaction was incubated at 30ºC for 12 hr. Crude eyestalk extract and PBS were used as positive and negative controls, respectively. The reaction was stopped by washing 3 times with cold normal saline containing 0.001% phenylmethylsulfonyl fluoride (PMSF) The sliced ovarian tissue was homogenized in 500 µl of cold normal saline and divided into a 100 µl fraction for detection of newly synthesized proteins and a 400 µl fraction for newly synthesized vitellin.

Newly synthesized proteins

The fraction of 100 µl for detection of newly synthesized proteins was precipitated with 400 µl of saturated ammonium sulphate, then centrifuged at 16,000xg for 10 min. The precipitate was washed with 300 µl of cold normal saline and resuspended in 150 µl of cold normal saline. A suspension of 50 µl was divided for determination of protein (Lowry *et al.*, 1951) and the other 100 µl was measured for radioactivity (^{14}C leucine). Percentage inhibition of protein synthesis was calculated according to the equation below;

% Inibition = (100x radioactivity of sample in dpm)/radioactivity of positive control in dpm)

Newly synthesized vitellin

The fraction of 400 µl (prepared as previously described in the GIH activity assay) was added to 50 µl of anti-vitellin and incubated for 1 hr. The reaction was then added with 50 µl of 10% protein A, prepared according to Harlow and Lane (1988). The mixture was incubated on ice for 30 min. The reaction was centrifuged at 10,000xg at 4ºC for 1 min. The sediment was washed with 0.5 ml lysis buffer (150 mM NaCl, 1.0% NP-40, 50mM Tris, pH 8.0). After centrifugation, 50 µl of sample buffer (2% SDS, 100mM DTT,60mM Tris, pH 6.8) was added to the sediment, heated at 85ºC for 10 min and centrifuged at 4ºC for 1 min. The supernatant was added to 950 ml PBS and mixed with 10 ml toluene-and xylene-based scintillators (toluene 1 l, tritonX-100 333 ml, PPO 4 g, POPOP 0.1 g) and counted for the radioactivity of ^{14}C-leucine in the newly synthesized vitellin by a liquid scintillation counter.

Results

Separation of the neuropeptide hormones from eyestalks

The supernatant of the eyestalk extract was separated on a µBondapak-Phenyl column into 8 peaks as shown in Figure 1. Peaks 3, 5 and 6 were large peaks and corresponded to those containing CHH and GIH as previously described (Aguilar *et al*., 1992). The protein concentration of peaks 3, 5 and 6 was too low to separate by polyacrylamide gel electophoresis; however, they were collected for CHH and GIH biological assays.

CHH activity

The fraction from peak 5 increased the glucose level in haemolymph significantly above that in the control group (SPSS analysis at 95% confidence) while the fraction from peak 6 showed no significant activation of the glucose level compared with the control group (no significant difference at 95% confidence). In contrast, the fraction from peak 3 showed no CHH activity (Table 1)

Purification of vitellin and antibody preparation

Homogenized ovaries of *P. merguiensis* were separated on Superose HR10/30 and the protein concentration in each fraction was determined. The major protein peak was found between fractions 5-7, (Figure 2). Each fraction was separated by 7% native gel electrophoresis and a vitellin band was found in fractions 5 and 6 (Figure 3C). Vitellin is a lipoglycoprotein, therefore the vitellin was identified by staining for carbohydrate and lipid

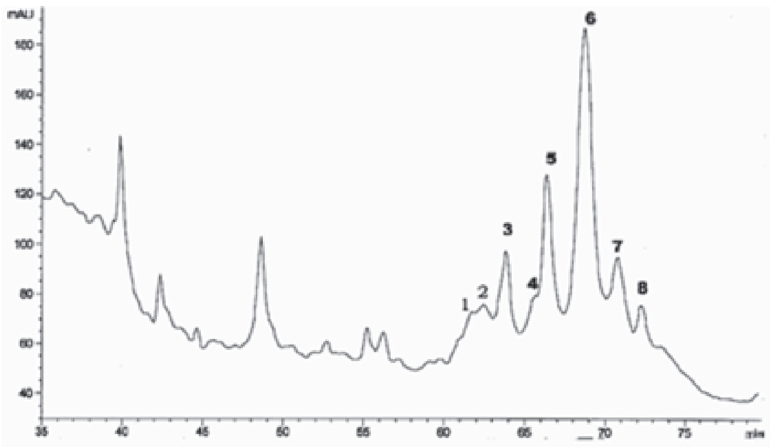

Figure 1. Separation of eyestalk extract from *P. merguiensis* by µBondapak-Phenyl column (9 mm i.d., 10 µm particle size, Waters). The linear gradient from 30%A (0.1 % TFA) to 80% B (0.1% TFA in 60% acetonitrile) in 80 min. Fractions of 3 ml each were collected.

Table 1. CHH activity of the peaks 3, 5 and 6 of the eyestalk extract separated by µBondapak-Phenyl column. Each sample was from a pool of 5 eyestalks separated by µBondapak-Phenyl column.

Sample	Control (n=5) Mean±SD	Peak 3(n=5) Mean±SD	Peak 5(n=5) Mean±SD	Peak 6(n=5) Mean±SD
Glucose (mg/dl)	6.237±0.677	5.347±1.105	*8.058±0.89	6.87±0.69

(* significantly different from the control at 95% confidence)

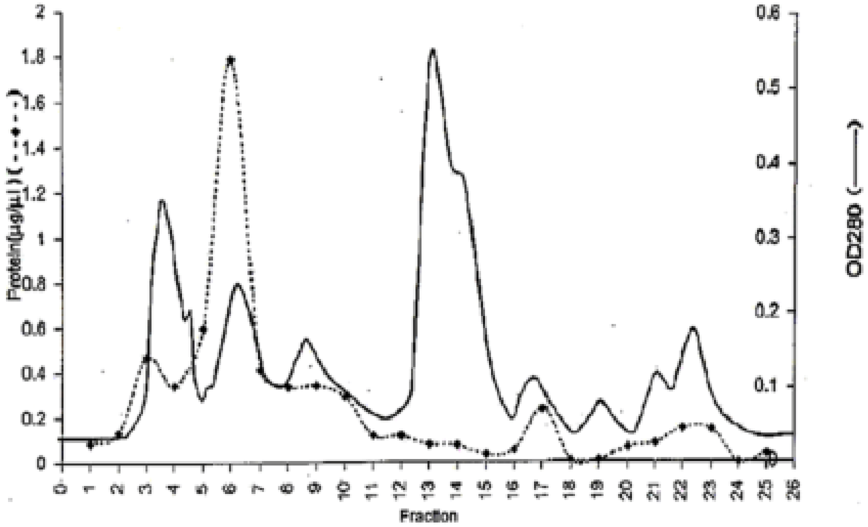

Figure 2. Profile of a crude extract of ovaries of *P. merguiensis* separated on Superose HR 10/30. The column was eluted with PBS containing 0.001% PMSF (phenylmethylsulfonyl fluoride) at a flow rate of 0.5 ml/min and 1 ml fractions were collected.

with PAS and Sudan Black B, respectively. Carbohydrate and lipid were detected on the protein band of fractions 5 and 6 (Figure 3A and 3B).

The anti-vitellin antibody of *P. merguiensis* was used to detect ovarian vitellin. Western blotting analysis (Figure 4) was performed after separating the samples on 7% native gel electrophoresis. The anti-vitellin showed a strongly positive band of vitellin in the crude extract of the ovaries and in the fraction 6. As the anti-vitellin can detect vitellin in the ovary, it can be used for immuno-precipitation of vitellin in a GIH activity assay. Furthermore, the anti-vitellin could be used to determine the maturation of the shrimp's ovary by quantifying the amount of vitellin. Since the amino acid sequence of vitellin is quite conserved, the anti-vitellin may be used to identify and quantify the vitellin in other species of shrimps.

GIH activity

Newly synthesized total proteins

The fractions of peaks 3, 5 and 6 from the µBondapak-Phenyl column were investigated for GIH activity. The eyestalk extract and PBS were used as positive and negative controls, respectively. The percentages of inhibition of the protein synthesis by the fractions of peaks 3, 5 and 6 were 75, 108 and 35, respectively, while that of the positive control was 100% (Table 1). Statistical analysis according to SPSS shows that the in-

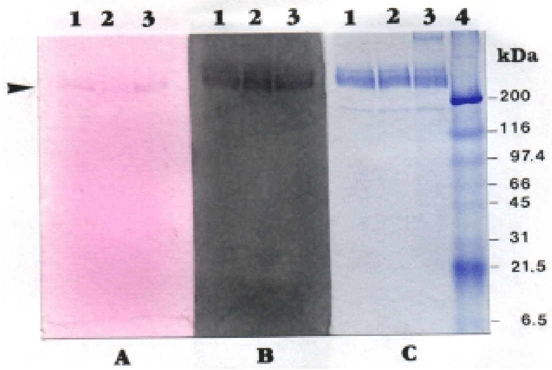

Figure 3. Vitellin separated on 7% native polyacrylamide gel
A: vitellin of *P. merguiensis* stained with PAS; lanes 1 and 2 vitellin fractions 5 and 6 (15 μg), lane 3 crude extract of ovaries (125 μg); B: vitellin of *P. merguiensis* stained with Sudan Black B; lanes 1 and 2 vitellin fraction 5 and 6 (5 μg), lane 3 crude extract of ovaries (10 μg); C: vitellin of *P. merguiensis* stained with Coomassie Blue; lanes 1 and 2 vitellin fraction 5 and 6 (5 μg), lane 3 crude extract of ovaries (10 μg), lane 4 standard molecular weight markers.

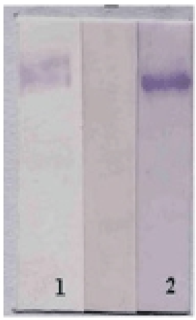

Figure 4. Western blotting analysis of vitellin after separation by 7% native gel electrophoresis., V_n = vitellin, lane 1 crude extract of mature ovaries of *P. mergiuensis* (5 μg), lane 2 purified vitellin (5 μg).

hibition of the fractions of peaks 3 and 5 were not different from the positive control (95% confidence) while that of peak 6 was not different from the negative control.

Newly synthesized vitellin

The eyestalk extract and PBS were used as positive and negative controls respectively for inhibition of vitellin synthesis. The fractions of peak 5 and 6 showed inhibition of vitellin synthesis at 94% and 71%, respectively (Table 2) whereas the fraction of peak 3 showed stimulation of vitellin synthesis at 116%. The comparison of the percentage inhibition of the newly synthesized total protein to the percentage inhibition of the newly synthesized vitellin of the samples of peaks 3 was 75% to -116%. This means that the fraction of peak 3 inhibited other protein synthesis except vitellin synthesis whereas the fraction of peak 5 and 6 inhibited protein synthesis including vitellin.

Discussion

The partially purified vitellin from the ovaries of *P. merguiensis* was used to produce the anti-vitellin antibody. In western blotting this antibody specifically bound to vitellin so it could

Table 2. Percentage inhibition of newly synthesized proteins and newly synthesized vitellin. Each sample was a pool of 10 eyestalks separated by the µbondapack phenyl column.

Sample	Control (-) (n=2)	Control (+) (n=2) Mean±SD	Peak 3 (n=2) Mean±SD	Peak 5 (n=2) Mean±SD	Peak 6 (n=2) Mean±SD
% inhibition of protein synthesis	0	100±15.9	75.18±11.5	108.39±13.5	34.98±36.4
% inhibition of vitellin synthesis	0	100±3.9	-116.15±32.3	93.99±37.3	70.68±14.8

be used in the GIH assay. The separation of the crude eyestalk extract on a RP-HPLC, µBondapack-Phenyl column produced 8 peaks, of which peaks 3, 5 and 6 were similar to those reported by Aguilar *et al*. (1992) and Huberman *et al*. (1995). It therefore seems reasonable to conclude that these peaks belong to the CHH/MIH/GIH family as proposed by Aguilar *et al*. (1992) and Huberman *et al*. (1995). A sample from the peak 3 fraction produced a 75% inhibition of new protein synthesis in the ovary but a 116% stimulation of new vitellin synthesis. Quackenbush (1992) found that the H2 peak (Huberman *et al*., 1989) separated from the extract from *Procambarus bouvieri* by the µBondapack-Phenyl column had MIH activity and stimulated yolk protein synthesis by as much as 300%. Peak 3 in this experiment and the Huberman' H2 peak behaved very similarly as both had two biological activities. Peak 3 had both GIH and GSH-like activities, but unfortunately we did not test for MIH activity. This study confirms that the prawn sinus gland contains a neuropeptide that stimulates yolk protein synthesis *in vitro*. (Quackenbush, 1992). Therefore, it may be possible to use GSH to stimulate ovarian maturation of *P. merguiensis* instead of eyestalk ablation. Fraction from peak 5 produced significant inhibition of both total protein synthesis and vitellin synthesis. This is an indication of the presence of GIH activity of Peak 5 while Peak 5 also had significant CHH activity. Quackenbush (1992) also found GIH activity from the peptide separated from *Procambarus bouvieri* (Huberman *et al*., 1989). Khayat *et al*. (1998) reported that CHH from *Penaeus japonicus* also provoked inhibition of protein synthesis in *Penaeus semisulcatus*. These findings lead to the conclusion that peak 5 could have both CHH and GIH activity. Peak 6 had slight CHH activity while the percentage of the inhibition of the total protein synthesis and the vitellin synthesis was 35% and 71%, respectively. This indicates that peak 6 specifically inhibited the vitellin synthesis. In conclusion, the sinus gland of *P. merguiensis* contain the substances which have CHH, GIH and GSH like activities.

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