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Production of Monoclonal Antibodies Specific to Eyestalk Neuropeptides of *Penaeus monodon* using Sinus Gland Section and Immunosuppression Technique

Nanthika Panchan[a,b], Paisarn Sitigorngul[c*], Parin Chaivisuthangkura[c], Siwaporn Longyant[c], Weerawan Sithigorngul[c] and Amorn Petsom[a,b]

[a] Biotechnology Program, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand.
[b] Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok 10330, Thailand.
[c] Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok 10110, Thailand.

* Corresponding author, E-mail: paisarn@swu.ac.th

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ABSTRACT: Immunosuppression method was utilized for immunization in order to generate monoclonal antibodies specific to neuropeptides in the eyestalk of *Penaeus monodon*. Selection of peptide specific monoclonal antibodies was done by immunohistochemistry against eyestalk sections. Four monoclonal antibodies bound to sinus gland and neurons in the eyestalk were isolated. All of these antibodies bound to putative peptides in different fractions of eyestalk extract separated by RP-HPLC. The antibodies were used to monitor the presence of the peptides during purification of sinus gland extract by RP-HPLC using dot-ELISA method. Crustacean hyperglycemic hormones (Sgp I, III and IV) were isolated and identified with SG 24, 26 and 293 monoclonal antibodies respectively. An unknown peptide with molecular mass of 9127.56 daltons was identified with SG 782 antibody. The N-terminal sequencing of the first 20 residues revealed that the sequence of this peptide is a novel peptide sharing no sequence identity to any known crustacean or vertebrate.

KEYWORDS: immunohistochemistry, monoclonal antibody, neuropeptide, *Penaeus monodon*, sinus gland.

INTRODUCTION

In decapod crustacean, the neuroendocrine system in the eyestalk consists of the X-organ located in the medulla terminalis (MTXO) and the sinus gland, a neurohaemal organ, where several hormones are released into haemolymph[1]. Red pigment concentrating hormone (RPCH) was the first hormone isolated and sequenced from the eyestalk of *Pandarus borealis*[2] followed by pigment dispersing hormone (PDH)[3] . Larger peptide hormones including crustacean hyperglycemic hormone (CHH)[4], molt-inhibiting hormone (MIH)[5] and vitellogenesis inhibiting hormone (VIH)[6] or gonad inhibiting hormone (GIH) were identified in crab *Carcinus maenas* and lobster *Homarus americanus*. These three peptides consist of 72-78 amino acid residues in length and are similar in amino acid sequences, including six conserved cysteine residues that form three disulfide bonds. They were called CHH/MIH/VIH(GIH) family[1]. Other members of this peptide family have been identified in *Cancer pagurus*. These include mandibular organ-inhibiting hormone (MOIH) which inhibits methyl farnesoate synthesis in mandibular organ[7], and a CHH/MIH/GIH (CMG) peptide which is structurally similar to a CHH identified in *Penaeus monodon*[8]. With the development of automated microsequencing and molecular cloning, many peptides in this family have been identified in various species[9].

Recently, eight isoforms of FMRFamide-like peptide family were identified in *Macrobrachium rosenbergii*[10,11]. In *P. monodon*, an economically important species for farming in Asian countries and Australia, various neuropeptides from the eyestalk have been identified, including 5 isoforms of CHH[12], CMG peptide[8], MIH[13], 7 isoforms of FMRFamide[14] and 4 isoforms of NPY/PP[15]. Identification has been through sequencing of cloned eyestalk cDNAs or through immunological identification, peptide isolation and sequencing. Recently, another family of neuropeptide "allatostatin" was identified in the nervous system of *P. monodon* using antiserum against the cockroach-type allatostatin[16], and the allatostatin-like peptide was immunohistochemically located in the eyestalk as well[17]. In *Ascaris suum*, new neuropeptides were identified using crude neuronal tissue extract as an immunogen for generating monoclonal antibodies. Hybridoma clones producing antibodies specific to a novel subset of neurons were then identified by immunohistochemistry. A monoclonal antibody that

recognized only a single neuron in the nervous system was subsequently used for peptide isolation. This approach yielded a unique peptide of molecular weight of 11,542 Da that was present in only a single neuron[18].

In this study we have used a similar approach, by generating monoclonal antibodies specific to eyestalk neuropeptides of *P. monodon* using sinus gland section and immunosuppression technique and selecting hybridoma clones by immunohistochemistry on the eyestalk sections. Characterization and specificity of monoclonal antibodies were identified by dot-ELISA[19] of eyestalk peptide fractions separated by one step RP-HPLC. Selected monoclonal antibodies were used for monitoring the peptide fractions during the purification processes. We were able to isolate and partially identify an eyestalk neuropeptide whose N-terminal sequence reveals a new sequence that shares no sequence identity with any peptide previously reported.

Methods

Production of Monoclonal Antibodies against Sinus Gland Peptides

Antigen Preparation

Eyestalks from live adult female *P. monodon* (at resting stage of ovarian development) were dissected, fixed and processed for paraffin serial sectioning (50 μm thickness). The sections were dewaxed and the position of sinus gland was located by immunohistochemistry of one in every ten sections using mouse anti-T+ antiserum (T+ isYANAVQTVamide : the putative C-terminal amide of crustacean hyperglycemic hormone (CHH) of *Macrobrachium rosenbergii*)[20]. The sinus gland from each section was dissected under the microscope then washed and homogenized with phosphate buffered saline (PBS) and stored at –70°C until use.

Immunosuppression and Immunization

Thoracic ganglia of *P. monodon* were dissected and fixed in Bouin fixative overnight. After washing thoroughly, the ganglia were homogenized in PBS, mixed with complete Freund's adjuvant (1:1) and injected to mice at approximately 0.5 ganglion/mouse. Three days after injection mice were injected with 100 μl of cyclophosphamide (40 mg/kg) dissolve in PBS. Three weeks later mice were immunized 4 times at two weeks intervals with sinus gland homogenate mixed with incomplete Freund's adjuvant (1:1) at approximately 5 eyestalks/mouse. One week after the fourth injection, serum from each mouse was collected and tested for binding to the sinus gland by immunohistochemistry against eyestalk sections. Mice with high titer of specific antibody against sinus gland were used as spleen cell donors for hybridoma production.

Monoclonal Antibody Production

P3X myeloma cells was used as fusion partner of mouse spleen cells using the method developed by Kohler and Milstein[21] modified by Mossman et al.[22] The products of each fusion was plated into 30 micro-culture plates (96 wells)/fusion. After identifying wells containing desired clones by screening immunohistochemically (described below), the cells were recloned at least twice by the limiting dilution method.

Monoclonal Antibody Screening

Immunohistochemistry

Eyestalks from live adult female *P. monodon* were collected, the external shell dissected then fixed in Bouin's fixative containing 0.5% glutaraldehyde for 24 h before processing for paraffin sectioning. Serial sections (5 μm thickness) were prepared and processed for indirect immunoperoxidase staining using various monoclonal antibodies obtained from hybridoma conditioned media. Horseradish peroxidase labelled goat anti mouse IgG heavy and light chain specific (GAM-HRP) antibody diluted to 1:1000 with 10% calf serum in PBS was used as a secondary antibody. Peroxidase activity was revealed by incubation with 0.03% DAB and 0.006% hydrogen peroxide in PBS. Preparations were counterstained with eosin Y, dehydrated in a graded ethanol series, cleared in xylene and mounted in permount[20]. Positive reactions were visualized as brown coloration against pink background of eosin Y.

Characterization of Monoclonal Antibody

Dot ELISA of RP-HPLC Fractions from Sinus Gland Extract

A highly sensitive dot-ELISA adapted from Sithigorngul et al.[19] was used to determine the presence of immunoreactive peptides in the fraction of eyestalk extract after RP-HPLC separation during purification as described below. Briefly, an aliquot of each fraction (approximately equivalent to 20-40 eyestalks/spot) was mixed with 1 mg/ml bovine serum albumin (BSA) dissolved in PBS at a proportion that yield 1 μg BSA/spot. The mixture was then dried in the vacuum concentrator. Distilled water (1 μl/spot) was added to dissolve the mixture and then 1 μl of the mixture was transferred as a spot on a nitrocellulose membrane. After the membrane was air dried and baked at 60°C for 30 min, it was exposed overnight to glutaraldehyde vapor in a tightly sealed plastic box and then soaked in 0.2% glutaraldehyde dissolved in PBS for 15 min. The membrane was washed thoroughly with distilled water and immersed for 5 min in 5% Blotto (5% nonfat dry milk, 0.1% triton X-100 and 0.01% Thimerosal in PBS). The membrane was incubated for 8 hr in 1:100 dilution

of various monoclonal antibodies. After washing with 0.05% Blotto (4 X 15 min), the membrane was then incubated for 8 h in GAM-HRP diluted at 1:1000 with 5% Blotto. The membrane was washed as before and then developed in a substrate solution (0.006% hydrogen peroxide, 0.03% DAB, and 0.05% $CoCl_2$ in PBS) for 3-5 min and washed thoroughly in distilled water.

Class and Subclass Determination:

Class and subclass of mouse immunoglobulins produced by hybridoma were determined by sandwich ELISA using Mouse MonoAb Kit (HRP; Zymed, USA).

Isolation of Immunoreactive Peptides

Preparation of Sinus Gland Extract

Sinus Gland Collection

Eyestalk was excised from live juvenile prawn (20-30g) and sinus gland was dissected in ice-cold 1.8% NaCl under the view of a dissecting microscope. The sinus gland was dropped immediately into ice-cold extraction solution (methanol: acetic acid: water / 90:1:9) and stored at –70°C until used.

Peptide Extraction

Approximately 2500 sinus glands were homogenized in 50 ml extraction solution on ice. After incubating at 4°C for 2 hr, the sample was centrifuged at 10,000 g for 30 min. The supernatant and the pellet were separately re-extracted with 25 ml extraction solution. After combining the supernatants, methanol and acetic acid was eliminated using a speed vacuum concentrator (Savant) then adjusting the solution to final concentration of 0.1% trifluoro acetic acid (TFA) for a final volume of 3 ml. The extract was then passed through C18 Sep-Pak cartridge and eluted with 50% acetonitrile 0.1% TFA. The eluate was concentrated with speed vacuum concentrator to eliminate acetonitrile then acetonitrile added to a final concentration of 10% (method adapted from Sithigorngul et al[14,15].

Chromatography

The sinus gland extract was centrifuged (10,000 ´g for 15 min) to remove undissolved material. Separations were performed on a Gilson HPLC system and monitored at 215 nm with Gilson 119 UV detector. In each step, 0.1% TFA or 0.1% heptafluorobutyric (HFBA) was used as a counter-ion. Three kinds of columns, namely C18 (Vydac), C8 and Cyano (Rainin Instruments) columns (size 4.6 X 250 mm), were used consecutively. Peptides were eluted with a linear gradient change of solvent B (80% acetonitrile plus counter-ion) over solvent A (water plus counter-ion) with gradient change of 1% solvent B/min, at 1 ml/min. Fractions were collected at 1 ml/min interval and aliquoted for identification of peptides recognized by different monoclonal antibodies separately.

Peptide Detection

Briefly, an aliquot of each sample (approximately content equivalent to 20 sinus glands/spot) was mixed with 1 mg/ml of BSA dissolved in PBS at a proportion that yielded 1 μg BSA/spot. The mixture was then dried in the vacuum concentrator. Distilled water was added to dissolve the BSA-peptide mixture, then 1 μl spotted on a nitrocellulose membrane and processed for dot ELISA as described above. The immunoreactive fractions were pooled and subjected to next step of purification until separated peaks were obtained.

MALDI-TOF MS

Approximately 10% of each purified fraction was used for molecular mass determination. Matrix-assist laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) analysis was carried out using α–cyano-4-hydroxycinnamic acid as matrix on a BIFLEX MALDI-TOF from Bruker-Franzen Analytik GMBH at the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University.

Microsequence analysis

Microsequence analysis of purified fractions by automated Edman degradation was performed by Rebecca Ettling at Biotechnology Resource Laboratory, Medical University of South Carolina, USA, using an Applied Biosystems Procise Sequencers.

Results

After the fourth immunization with sinus gland isolated from paraffin sections into three mice, antisera from two mice showed strong and specific staining of sinus gland and exhibited slight staining on different sub-populations of neurons. The two mice were used as spleen cell donors for hybridoma production. However the yields from both fusion were low, resulting in approximately 300-400 clones/fusion. Four established hybridoma clones producing specific monoclonal antibodies were isolated. All of these antibodies bound specifically to various sub-population of neurons in the eyestalk of *P. monodon* and sinus gland (Fig. 1, Table 1). Antibodies designated SG 24, SG 26 and SG 293 recognized approximately 20-35 neurons in the medulla terminalis X-organ complex (MTXO) and antibody SG 782 recognized only 2-3 MTXO neuronal cell bodies which were different from sub-populations recognized by the other three antibodies (Fig 1). On dot-ELISA tested against eyestalk extract after first step of RP-HPLC separation, three antibodies (SG 24, SG 26 and SG 293 antibodies) bound to different

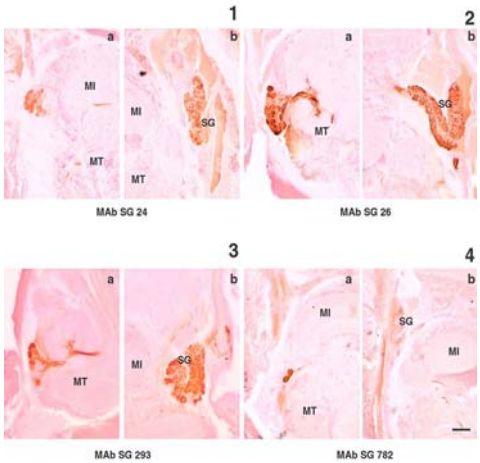

Fig 1. Immunohistochemical localization of the peptides recognized by monoclonal antibodies: (1) SG 24 (2) SG 26 (3) SG 293 (4) SG 782. All the antibodies recognized neuronal cell bodies in the MTXO (a) and sinus gland (b). MI = medulla interna, MT = medulla terminalis and SG = sinus gland.

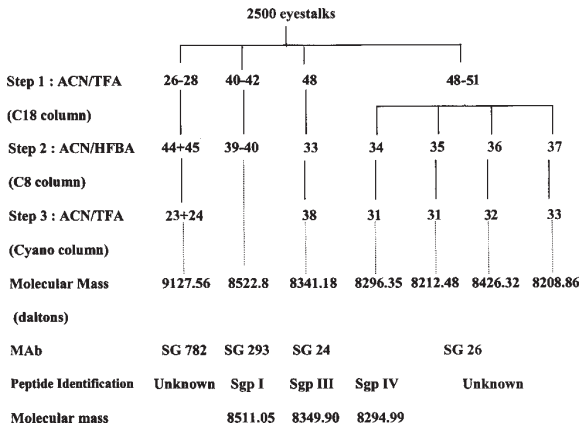

Fig 3. Purification steps of the peptides recognized by monoclonal antibodies. Putative CHHs identified by Davey et al[12] were compared with the peptides obtained at the bottom rows.

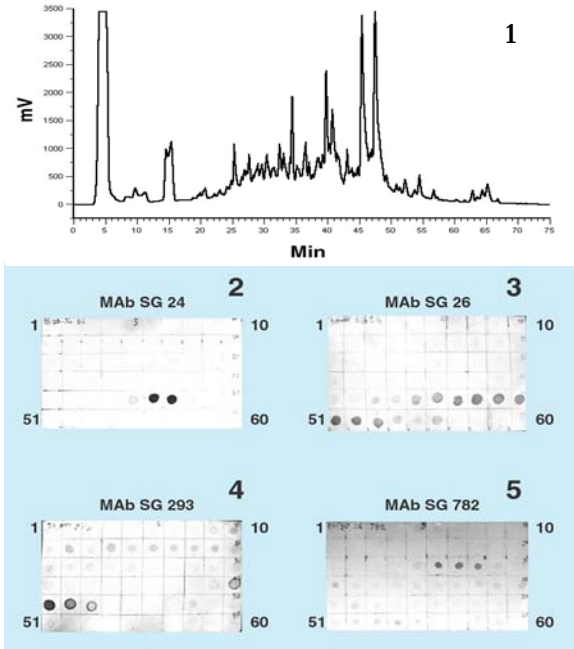

Fig 2. Separation of sinus gland extract and localized of the peptides recognized by monoclonal antibodies. (1) chromatogram of the extract at the first step of RP-HPLC separation. Dot-ELISA of the fractions treated with (2) SG 24 (3) SG 26 (4) SG 293 and (5) SG 782 antibodies. The fractions were collected at one minute intervals. The number indicated fraction numbers only at the first and last fractions of the top and bottom rows.

fractions except for two fractions (46-47) which were bound by SG 24 and SG 26 (Fig. 2). All fractions were also recognized by anti-T+ antiserum, which is the antiserum made against C-terminal peptide of CHH from *Macrobrachium rosenbergii*[19]. The other antibody (SG 782) bound to fractions 26-28 (Fig. 2). The peptides from these fractions were further purified by one or two more steps of RP-HPLC (Fig. 3) and using

Table 1. Characterization of monoclonal antibodies against sinus gland peptides.

Monoclonal Antibodies	Class & Subclass	Immunohistochemistry		Dot Blot, 1st step HPLC Fractions	Peptides (daltons)
		Neurons	Sinus gland		
SG 24	IgM	~20	+++	46-47 Sgp III (8349.9)	CHH (8341.18)
SG 26	IgG1	~35	+++	43-53 Sgp IV (8294.99)	CHHs 1(8296.35) 2(8426.32)* 3(8212.48)* 4(8208.86)*
SG 293	IgM	~30	+++	40-43 Sgp I (8511.05)	CHH (8522.8)
SG 782	IgM	2-3	+	26-28	Unknown (9127.56)

* amino acid sequence was not determined

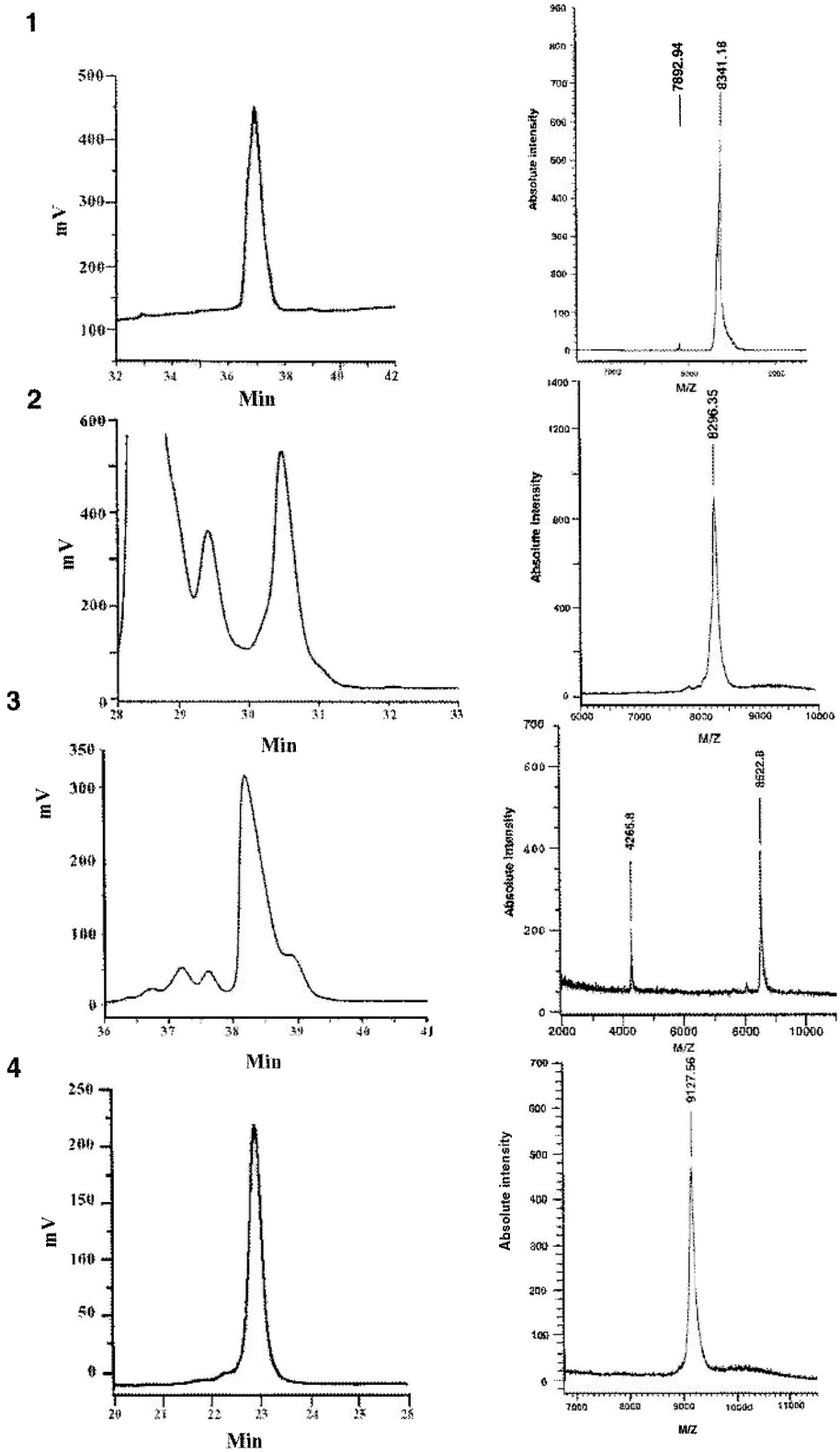

Fig 4. Chromatograms and MALDI TOF MS analysis of each purified peptide at the last step of purification. (1) SG 24 (2) SG 26 (3) SG 293 and (5) SG 782.

1				
SG 24	ANFDPSCAGV	YNRELLGRLS	RLCDDCYNVF	REPKVATXCR
	NXCFYNPVFV	QCLXY		
Sgp III	ANFDPSCAGV	YNRELLGRLS	RLCDDCYNVF	REPKVATECR
	NNCFYNPVFV	QCLEYLIPAD	LHEEYQAHVQ	TVGK
2				
SG 26-1	SLFDPACTGI	YDRQLLGKLG	RLCDDCYNVF	REPKVATGCR
	XNCYYNLIFL			
Sgp IV	SLFDPACTGI	YDRQLLGKLG	RLCDDCYNVF	REPKVATGCR
	SNCYYNLIFL	DCLEYLIPSH	LQEEHMEALQ	TVGK
3				
SG 293	SLFDPSCTGV	FDRQLLRRLS	RVCDDCFNVF	REPNVAXQCR
Sgp I	SLFDPSCTGV	FDRQLLRRLS	RVCDDCFNVF	REPNVATECR
	SNCYNNEVFR	QCMEYLLPAH	LHEEHRLAVQ	MVGK
4				
SG 782	AKEAA E(A)TKEV AETKP(K) Q(G)S(EA)E(K)XK			
Unknown				

Fig 5. Amino acid sequences of the 4 peptides (1-4) isolated by each monoclonal antibodies and comparison with the known peptides. The letters in the parenthesis are minor amino acid presence in that cycle. X = unknown. Underlined is the different sequence.

monoclonal antibodies specific to those fractions to monitor the peptides during purification until the relatively pure peptides were obtained (Fig. 4). All the obtained peptides that bound to SG 24, SG 26 and SG 293 antibodies were cross-reacted with anti-T+ antiserum. MALDI-TOF analysis revealed that their molecular masses were range from 8200-8500 daltons which are the range of CHHs molecular mass. Only three peptides, one from fractions 40-42 (recognized by SG 293), one from fractions 46-47 (recognized by SG 24) and one of the four peptides from fractions 48-51 recognized by SG 26 antibody (SG 26-1), were subjected to sequence analyses (Fig. 5). The N-terminal sequence of the three peptides range from 37-50 residues revealed similarity to the N-terminal sequence of CHHs, namely Pem-Sgp I, Pem-Sgp III and PemSgp IV identified by Davey et al[12] (Table 1 and Fig. 5).

The molecular mass of peptide isolated from fractions 26-28, recognized by SG 782 antibody, was 9127 daltons. Sequence analysis of the first 20 residues at the N-terminus, revealed a new sequence which shares no similarity to any peptides previously reported (Fig. 5). Since the yield of the peptide was low, approximately 5 pmol, it was impossible to identify the whole sequence and the amino acids at several positions were still uncertain.

Discussion

We were able to isolate a monoclonal antibody specific to an unknown peptide along with monoclonal antibodies specific to various isoforms of CHHs, by using mice immunized with fixed sinus gland of *P. monodon* isolated from paraffin section of eyestalks. The yield of hybridoma from two fusions were quite low. This reflected the low number of positive antibodies obtained from this experiment. As such, it reduced the opportunity to obtain other antibodies specific to other unknown and/or known peptides such as MIH[13], CMG peptide[8], FMRFamide peptide family[14] and NPY/PP family[15]. Therefore, increasing the yield of hybridoma production by any means would increase the opportunity to obtain antibodies to the rare peptides.

CHH seems to be in high abundance in the sinus gland since the majority of the monoclonal antibodies generated are against CHHs. Two monoclonal antibodies specific to different isoforms of CHHs were obtained, SG 293 antibody recognized only Sgp I and SG 24 antibody recognized only Sgp III, while the other antibody SG 26 recognized most of CHHs except for Sgp I. However, subpopulations of CHH containing neurons recognized by 3 antibodies were slightly different, indicating that most of the CHH containing neurons could selectively express some of the CHH isoforms.

Interestingly, an antibody specific to an unknown peptide (SG 782) is a IgM, but it could still be used to monitor the peptide during purification, and the specific peptide was isolated. N-terminal sequence of the peptide revealed a novel sequence which did not show any similarity to known peptide families of crustacean (including MIH and VIH which are in the range of similar molecular mass) and other organisms. A similar approach successfully isolated a monoclonal antibody which recognized only a single neuron of *Ascaris*. Using an immunosuppressed mouse immunized with crude homogenate of dissected nervous tissue, a 11 kD peptide was isolated. The antibody was used to monitor the peptide during the purification processes[18]. This evidence reflects the efficiency of mouse immune system which responds to very minute amounts of antigen in the crude extract. The immunosuppression technique could enhance the response to these low concentration antigens which may be produced from only 2-3 neurons of the whole eyestalk. The full sequence of the isolated peptide could not be completely identified in this experiment since the peptide concentration in the eyestalk was very low. Due to the low concentration, further attempts to purify the peptide may be laborious. As another approach, the limited peptide sequence generated allows for molecular cloning of the gene specifying this peptide using cDNA library of *P. monodon* eyestalk which is in progress.

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REFERENCES

1. Keller R (1992) Crustacean neuropeptides: structure, functions and comparative aspects. *Experientia* **48**, 439-48.
2. Fernlund P, Josefsson L (1972) Crustacean color change hormone: amino acid sequence and chemical synthesis. *Science* **177**, 173-5.
3. Fernlund P (1976) Structure of a light-adapting hormone from the shrimp, *Pandalus borealis*. *Biochim Biophys Acta* **439**, 17-25.
4. Kegel G, Reichwein B, Weese S, Gaus G, Katalinic JP and Keller R. (1989) Amino acid sequence of the crustacean hyperglycemic hormone (CHH) from the shore crab *Carcinus maenas*. *FEBS letts* **225**, 10-4.
5. Webster SG (1991) Amino acid sequence of putative molt-inhibiting hormone from the crab *Carcinus maenas*. *Proc R Soc Lond B* **224**, 247-52.
6. Soyez D, Le Caer JP, Noel PY and Rossier J (1991) Primary structure of two isoforms of the vitellogenesis inhibiting hormone from the lobster, *Homarus americanus*. *Neuropeptides* **20**, 25-32.
7. Wainwright G, Webster SG, Wilkinson MC, Chung JS and Reese H (1996) Structure and significance of mandibular organ-inhibiting hormone in crab, *Cancer pagurus*. *J Biol Chem* **22**, 12749-54.
8. 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* **244**, 145-56.
9. Lacombe C, Greve P and Martin G (1999) Overview on the sub-grouping of the crustacean hyperglycemic hormone family. *Neuropeptides* **33**, 71-80.
10. Sithigorngul P, Saraithongkum W, Jaideechoey S, Longyant S and Sithigorngul W (1998) Novel FMRFamide-like neuropeptides from the eyestalk of the giant freshwater prawn *Macrobrachium rosenbergii*. *Comp Biochem Physiol* **120(B)**, 587-95.
11. Sithigorngul P, Saraithongkum W, Longyant S, Panchan N, Sithigorngul W and Petsom A (2001) Three more novel FMRFamide-like neuropeptide sequences from the eyestalk of the giant freshwater prawn *Macrobrachium rosenbergii*. *Peptides* **22**, 191-7.
12. Davey ML, Hall MR, Wills RH, Oliver RWA, Thurn MJ and Wilson KJ (2000) Five crustacean hyperglycemic family hormones of *Penaeus monodon*: Complementary DNA sequence and identification in single sinus glands by Electrospray Ionization-Fourier Transform Mass Spectrometry. *Mar Biotechnol* **2**, 80-91.
13. Krungkasem C, Ohira T, Yang W-J, Abdullah R, Nagasawa H and Aida K (2002) Identification of two distinct molt-inhibiting hormone-related peptides from the giant tiger prawn *Penaeus monodon*. *Mar Biotechnol* **4**, 132-140
14. Sithigorngul P, Pupuem J, Krungkasem C, Longyant S, Chaivisuthangkura P, Sithigorngul W and Petsom A (2002a) Seven novel FMRFamide-like neuropeptide sequences from the eyestalk of the giant tiger prawn *Penaeus monodon*. *Comp Biochem Physiol* **131B**, 325-37.
15. Sithigorngul P, Pupuem J, Krungkasem C, Longyant S, Panchan N, Chaivisuthangkura P, Sithigorngul W and Petsom A (2002b) Four novel PYFs: members of NPY/PP peptide superfamily from the eyestalk of the giant tiger prawn *Penaeus monodon*. *Peptides* **23**, 1895-906.
16. Duve H, Johnsen AH, Scott AG and Thrope A (2002) Allatostatins of the tiger prawn, *Penaeus monodon* (Crustacea: Penaeidae). *Peptides* **23**, 1039-51.
17. Panchan N, Bendena WG, Bowser P, Lungchukiet P, Tobe SS, Sithigorngul W, Chaivisuthangkura P and Rangsiruji A, et al (2003) Immunolocalization of allatostatin-like neuropeptides and their putative receptor in eyestalks of the tiger prawn, *Penaeus monodon*. *Peptides* **24**, 1563-70.
18. Sithigorngul P, Nanda JC and Stretton AOW (2003) A strategy for isolating rare peptides: isolation and sequencing of a large peptide present in a single neuron of the nematode *Ascaris suum*. *Peptides* **24**, 1025-33.
19. Sithigorngul P, Stretton AOW and Cowden C (1991) A versatile dot-ELISA method with femtomole sensitivity for detecting small peptides. *J Immunol Methods* **141**, 23-32.
20. Sithigorngul P, Panchan C, Vilaivan T, Sithigorngul W and Petsom A (1999) Immunochemical analysis and immunocytochemical localization of crustacean hyperglycemic hormone from the eyestalk of *Macrobrachium rosenbergii*. *Comp Biochem Physiol* **124B**, 73-8.
21. Kohler G and Milstein C (1976) Derivation of specific antibody-producing tissue culture and tumor cell fusion. *Eur J Immunol* **6**, 511-9.
22. Mosmann TR, Bauman R and Williamson AR (1979) Mutation affecting immunoglobulin light chain secretion by myeloma cells:I: Functional cell analysis by cell fusion. *Eur J Immunol* **9**, 511-6.