การผลิตโมโนโคลนอลแอนติบอดีต่อนิวโรเพปไทด์ฮอร์โมนในก้านตากุ้งกุลาดำ

Penaeus monodon

นางสาวนันทิกา ปานจันทร์

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PRODUCTION OF MONOCLONAL ANTIBODY AGAINST EYESTALK NEUROPEPTIDE HORMONES IN THE BLACK TIGER PRAWN *Penaeus monodon*

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นันทิกา ปานจันทร์ : การผลิตโมโนโคนอลแอนติบอดีต่อนิวโรเพปไทด์ฮอร์โมนในก้านตาของกุ้งกุลาดำ *Penaeus monodon* (PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST EYESTALK NEUROPEPTIDE HORMONES IN THE BLACK TIGER PRAWN, *Penaeus monodon*) อ. ที่ปรึกษา : รศ.ดร.อมร เพชรสม อ.ที่ปรึกษาร่วม : รศ.ดร.ไพศาล สิทธิกรกุล, 122 หน้า. ISBN 974-17-6215-1.

การศึกษาเพื่อพยายามพิสูจน์ทราบโครงสร้างเพปไทด์ฮอร์โมนชนิดต่างๆ ในก้านตากุ้งกุลาดำ เริ่มด้วยการตรวจหานิวโรเพปไทด์ที่ตรวจพบในแมลงต่างๆ ได้แก่ Allatostatin (AST) เพื่อใช้เป็นแนวทางในการพิสูจน์ทราบนิวโรเพปไทด์ฮอร์โมนในก้านตากุ้งกุลาดำ โดยใช้แอนติบอดี 4 ชนิดต่อ allatostatin (AST) ตรวจหาสารคล้าย AST ในก้านตากุ้งกุลาดำ โดยวิธี immunohistochemistry พบตัวเซลล์ประสาทในบริเวณ lamina ganglionalis ด้านหน้าของ medulla externa (ME) และบริเวณด้านหน้าและด้านหลังของ medulla terminalis (MT) และพบใยประสาทในบริเวณ ME MT ต่อมไซนัสและเส้นประสาทตา ไม่พบในบริเวณ medulla interna (MI) พบตัวเซลล์ประสาทมากกว่าร้อยเซลล์ใน X-organ จากแหล่งที่พบสารคล้าย AST ชี้ให้เห็นว่าสารนี้ทำหน้าที่เป็น neurotrasmitter และ/หรือ neuromodulator จากการใช้แอนติซีรัมต่อ *Drosophila* receptor (Dar-2) พบว่าจับกับโปรตีนหนึ่งชนิดในสารสกัดโปรตีนจากก้านตากุ้งกุลาดำ ซึ่งมีขนาดเท่ากับที่พบในสารสกัดโปรตีนจาก *Drosophila* และพบ AST receptor ในบริเวณต่อมไซนัสของกุ้งกุลาดำวัยอ่อนและโตเต็มวัย จากความรู้นี้แสดงให้เห็นว่าเป็นการพบ neuroreceptor ครั้งแรกในต่อมไซนัสของสัตว์ครัสตาเซียน ซึ่ง AST อาจทำหน้าที่โดยตรงเป็น neuromodulator ในต่อมไซนัส ในกุ้งกุลาดำวัยอ่อนเพศผู้ พบ AST receptor ที่กลุ่มเซลล์ประสาทในบริเวณ MT แต่ไม่พบในกุ้งเพศเมีย นัยสำคัญของความจำเพาะของแหล่งที่พบ receptor ในกุ้งเพศผู้และเพศเมียยังไม่ทราบแน่ชัด แต่แน่นอนว่า AST มีบทบาทในระบบประสาทของก้านตากุ้ง

อีกแนวทางการหนึ่งสำหรับใช้แอนติบอดีสำหรับพิสูจน์ทราบนิวโรเพปไทด์ที่ยังไม่มีการพิสูจน์ทราบมาก่อนโดยการสร้างและเลือกโมโนโคนอลแอนติบอดีต่อนิวโรเปปไทด์ในต่อมไซนัสของก้านตากุ้งกุลาดำ *P. monodon* ผลิตโดยการใช้ต่อมไซนัสที่แยกจากเนื้อเยื่อก้านตากุ้งที่แยกจาก parafin section ทำการคัดเลือกศึกษาคุณสมบัติโมโนโคนอลแอนติบอดีต่อต่อมไซนัส โดยวิธี immunohistochemstry และ dot-ELISA สามารถแยกโมโนโคนอลแอนติบอดีได้ 12 ชนิด พบว่า แอนติบอดีทั้งหมดทำปฏิกิริยากับต่อมไซนัสและตัวเซลล์ประสาทในก้านตากุ้ง และทำปฏิกิยากับนิวโรเพปไทด์ในแฟรคชั่นที่แตกต่างกันของสารสกัดจากต่อมไซนัสหลังจากแยกโดย RP-HPLC สามารถใช้ในการติดตามเพปไทด์ในระหว่างการทำให้บริสุทธิ์ของสารสกัดจากต่อมไซนัสโดย RP-HPLC และ dot-ELISA พบว่าสามารถแยกฮอร์โมนเพิ่มระดับน้ำตาลในเลือด (CHH) (Sgp-I, III และ IV) โดยใช้โมโนโคนอลแอนติบอดี SG24, 26 และ 293 เพปไทด์ที่ยังไม่ทราบชนิดที่มีน้ำหนักโมเลกุล 9127.56 ดาลตันแยกได้โดยใช้โมโนโคนอลแอนติบอดี SG782 จากลำดับกรดอะมิโน 20 หน่วยทางปลาย N พบว่าลำดับกรดอะมิโนของเพปไทด์นี้ไม่เหมือนกับลำดับกรดอะมิโนใดๆ ในสัตว์ครัสตาเซียนหรือสัตว์มีกระดูกสันหลัง นอกจากนี้ยังสามารถแยกโมโนโคลนอลแอนติบอดีที่จำเพาะต่อ PDH prepro-related peptide

ลายมือชื่อนิสิต.....

สาขาวิชา.....เทคโนโลยีชีวภาพ... ลายมือชื่ออาจารย์ที่ปรึกษา.....

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KEY WORD: EYESTALK/ MONOCLONAL ANTIBODY /NEUROPEPTIDE/ *Penaeus monodon*/ SINUS GLAND

NANTHIKA PANCHAN : PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST EYESTALK NEUROPEPTIDE HORMONE IN THE BLACK TIGER PRAWN, *Penaeus monodon*. THESIS ADVISOR : ASSOC. PROF. AMORN PETSOM, Ph.D. THESIS COADVISOR : ASSOC. PROF. PAISARN SITHIGORNGUL, Ph.D., 122 pp. ISBN 974-17-6215-1.

Identification of various neuropeptide hormone in the eyestalk of *Penaeus monodon* were performed by two approaches. Firstly a study of a known neuropeptide family found in various insect the allatostatin (AST) family. Immunohistochemical study on the eyestalk of *P. monodon* using 4 kinds of anti-allatostatin antisera revealed that AST-like immunoreactivity in various regions; neuronal bodies in the lamina ganglionalis, anterior to the medulla externa (ME) and in the anterior and posterior of the medulla terminalis (MT). Neuronal processes in neuropiles of the ME, MT, sinus gland and nerve fibers in the optic nerve were also recognized. No immunoreactivity in cell bodies or in nerve fibers was found in the medulla interna (MI). Strong AST-like immunoreactivity was found in hundreds of cells of the X- organ. The localization of AST-like peptides suggests that they function as neurotransmitters and/or neuromodulators. Antiserum to the *Drosophila* AST receptor (Dar-2) recognized a single protein in *P. monodon* eyestalk protein extracts that was identical in size to that found in *Drosophila* protein extracts. Using this antiserum the putative *P. monodon* AST receptor was localized to the sinus gland in both juvenile and adult eyestalks. To our knowledge this is the first demonstration of a neuropeptide receptor localized to the crustacean sinus gland. This suggests that ASTs may function directly on the sinus gland as a neuromodulator. In juvenile eyestalks, the putative AST receptor was also localized to neuronal X-organ cells of MT in males but not in females. The significance of this sex-specific receptor localization is unclear but emphasizes that ASTs function within the nervous system of the eyestalk.

In order to identify unknown neuropeptides in the eyestalk the second approach was performed. Monoclonal antibodies directed against sinus gland neuropeptides were generated from mice immunized with sinus glands dissected from paraffin sections of eyestalks from *Penaeus monodon*. Selection of peptide specific monoclonal antibodies was done by immunohistochemistry against eyestalk sections. Characterization and specificity of monoclonal antibodies were identified by immunohistochemistey and by dot-ELISA of sinus gland extract separated by one step RP-HPLC. Twelve 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. CHHs (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. Moreover several antibodied bound to the PDH-prepro-related peptide of various peptide were also isolated

Student's signature…….

Field of study…Biotechnology…. Advisor's signature……

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CHAPTER I

Introduction

Prawn aquaculture industry, particularly the black tiger prawn, *Penaeus monodon* has been expanded dramatically after the expansion of the technology of intensive marine shrimp farming during the last 10 years, especially in Asia countries such as Thailand, Taiwan, China and Indonesia. Thailand has become the leading country in shrimp production since 1991, providing considerable economic benefits for farmer and the diverse industries which service and support shrimp farming. More than 90% of *Penaeus monodon* culture production has been exported as frozen headless and with shell on. The value of exported shrimp products has become an important revenue for Thailand (Jenkins et al., 1999; Tookwinas, online). Even though the prawn culture techniques have been achieved but to date, this industry has faced various problems. One problem is an insufficient supply of broodstock for hatchery production especially, female. The broodstock comes from the capture fisheries and then are kept in captivity. In order to control female reproduction in captivity, the technique of eyestalk ablation is used to induce ovarian maturation. Eyestalk ablation destroys the X-organ which not only depletes the gonad-inhibiting hormone or vitellogenesis inhibiting hormone which control ovarian maturation but also other hormones that regulates various physiological activities.

The most commonly accepted hypothesis about ovarian development in most crustacean is that a gonad inhibiting hormone (GIH) is produced in the neurosecretory complexes in the eyestalk. This hormone apparently occurs in the non-breeding season and is absent or present only in low level during the breeding season. By inference, then, the reluctance of most penaeids to routinely develop mature ovaries in

captivity is a function of elevated levels of GIH, and eyestalk ablation lowers the high hemolymph titer of GIH (Bray and Lawrence, 1992)

The effect of eyestalk removal is not on a single hormone, GIH, but rather effects numerous physiological processes; carbohydrate metabolism, molting, heart rate, osmoregulation and color change (Belt, 1988; Keller, 1992) due to the fact that the X-organ-sinus gland system in eyestalk is a major source of various neuropeptides such as red pigment concentrating hormone (RPCH) (Fernlund and Josefsson, 1972), pigment dispersing hormone (PDH) (Fernlund, 1976), crustacean hyperglycemic hormone (CHH) (Kegel et al., 1989), molt-inhibiting hormone (MIH) (Webster, 1991), mandibular organ-inhibiting hormone (MOIH) (Wainwright et al., 1996) which regulated various physiological processes. The effect of eyestalk ablation to offspring produced by eyestalk-ablated females have been perceived to be poorer quality than those produce by wild-developed females. Indeed, in captive stocks, the following are sometimes observed: smaller spawn size (lower number of egg/spawn), lower hatching rate, lower survival to post larval stages, lower survival in growout, increased larval susceptibility to disease and higher mortality of eyestalk-ablated female (Bray and Lawrence, 1992).

Even though, the relation between removal of eyestalk of female on decapod crustacean and ensuring gonadal development was first discovered by Panouse (1943) but the progress of hormonal control studies about reproductive physiology of both male and female are slowly expanded. Hence, in order to induce ovarian development without destroying other hormonal system in eyestalk, the study of more neuropeptide hormones in eyestalk is importance to increase the knowledge on the regulation of

reproduction in decapod crustacean, especially *P. monodon* for improving the sustainability of prawn culture industry.

In order to identify various neuropeptide hormones in eyestalk of *P. monodon*, two approaches of this study were presented. The first approach was a preliminary identification the known peptide family involved reproduction in various insect, allatostatin (AST), in the eyestalk of *P. monodon* using four antisera against allatostatin and an antiserum against AST-receptor by immunohistochemistry. We are able to demonstrate that AST-like peptide and the putative AST receptor were localized in various regions of the eyestalk of *P. monodon* and this is the first demonstration of a neuropeptide receptor localized to the crustacean sinus gland.

Another approach was developed in order to identify unknown neuropeptide in the eyestalk of *P. monodon*. Random productions of monoclonal antibodies against eyestalk neuropeptides from fixed sinus gland isolated from eyestalk sections were performed. Then the monoclonal antibodies were identified by immunohistochemistry of eyestalk and dot-ELISA (Sithigorngul et al., 1991) of eyestalk peptide fractions separated by one step RP-HPLC. Selected monoclonal antibodies were used to monitor 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 previously reported peptide.

The following content of this thesis will be consist of Chapter II, a literature review about general biology of *P. monodon*, crustacean endocrinology system neuropeptides found in the eyestalk of crustacean. Chapter III will be a preliminary study of allatostatin-like peptide in the eyestalk. Chapter IV is generated monoclonal

antibodies against sinus gland neuropeptides from the sinus gland isolated from eyestalk section. Chapter V is monoclonal antibodies against other sinus gland neuropeptides with partially identified specificity. Chapter VI will be conclusion of the overall results of this studies.

CHAPTER II

Literature review

2.1 Biology of *Penaeus monodon* Fabricius, 1978

The taxonomic definition of the black tiger prawn is as following:

Phylum Arthropoda

Class Crustacea

Subclass Malacostraca

Order Decapoda

Suborder Natantia

Infraorder Penaeidea

Superfamily Penaeoidea

Family Penaeidae Rafinesque, 1815

Genus *Penaeus* Fabricius, 1798

Subgenus *Penaeus*

Species *monodon*

Distribution

Black tiger prawn, *Penaeus monodon*, is one of the largest penaeid prawns. The prawn *P. monodon* is widely distributes throughout the greater part of the Indo-Pacific region, ranging northward to Japan and Taiwan, eastward to Tahiti, southward

to Australia, and westward to Africa. In general, *P. monodon*, distributes from 30 °E to °155 E in longitude from 35 °N to 35 °S in latitude with the main fishing grounds located in tropical countries (Solis, 1988).

Morphology

A live *P. monodon* has the following characteristic coloration (Fig. 2.1): carapace and abdomen are transversely banded with red and white, the antennae are grayish brown, and the pereopods and pleopods are brown with crimson fringing setae. In shallow brackish waters or when cultured in ponds, the color changes to dark and, often, to blackish brown (Solis, 1988).

The shell is smooth, polished and glabrous. The rostrum extends beyond the tip of the antennular peduncle, is sigmoidal in shape, and possesses 6-8 dorsal and 2-4 ventral teeth, mostly 7 and 3, respectively. The Carapace is carinated with the adrostral carina almost reaching the posterior margin of the carapace. The gastro-orbital carina occupies the posterior one-third to one-half distance between the post-orbital margin of the carapace and the hepatic spine. The hepatic carina is prominent and almost horizontal. The antennular flagellum is subequal to or slightly longer than the peduncle. Exopods are presents on the first four pereopods but absent in the fifth. The abdomen is carinated dorsally from the anterior one-third of the fourth, to the posterior end of the sixth, somites. The telson has a median groove but without dorsolateral spinces (Fig. 2.2) (Farfante and Kensley, 1997; Solis, 1988).

Figure 2.1 The black tiger prawn, *P. monodon*, female (top) and male (bottom).

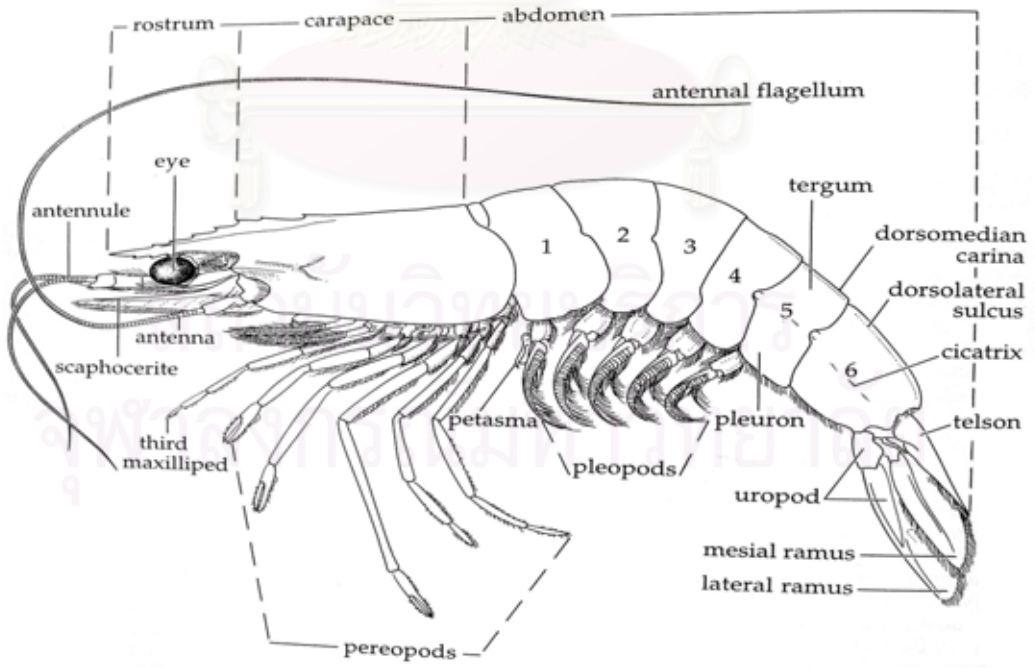


Figure 2.2 Lateral view of black tiger prawn, *P. monodon* (Farfante and Kensley, 1997)

External morphology of reproductive system

The *P. monodon* is heterosexual. The female attains a relative larger size than the male. The sexually mature prawn can be distinguished by the presence of the external genital organs: joined petasma, a pair of appendix masculina on the exopods of the second pleopods, and genital opening on the coxa of the fifth of pereopod for the male. In females, the thelycum is situated between the fourth and fifth pair of pleopods, consists of an anterior and a pair of lateral plates (Fig. 2.3). It receives the spermatophores during mating. In *P. monodon*, the thelycum is classified as closed type. The genital opening is on the coxa of the third pereopod (Farfante and Kensley, 1997; Solis, 1988).

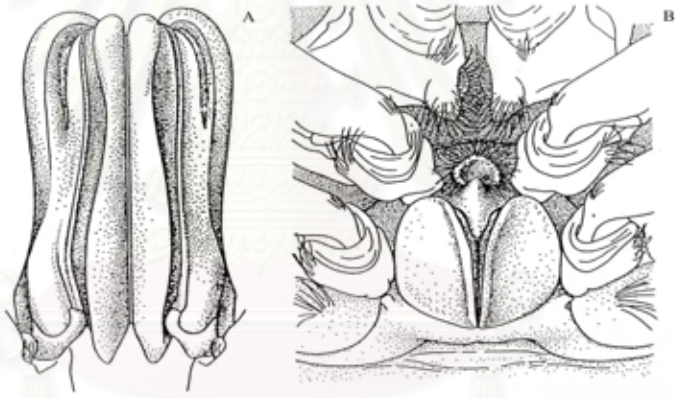


Figure 2.3 External genitalia of male, petasma (A) and female, thelycum (B) of *P. monodon* (Farfante and Kensley, 1997).

Morphological Development

Embryo. Eggs are spherical, yellowish green, and very minute, having diameter ranging from 0.27 to 0.31 mm with an average of 0.29 mm. Eggs tend to sink slowly in still water. Cleavage to 2-celled, 4-celled, morula, and embryonic nauplius stages occur approximately 0.5, 1, 1.8, and 11 hours, respectively after

spawning. The nauplius in each egg is observed to move intermittently before hatching (Solis, 1988).

Larva. The larval stage consists of 6 nauplius, protozoea, 3 mysis, and 3 or 4 megalopa substages, requiring about 1.5 days, 5 days, 4-5 days, and 15 days respectively, for development. Larvae exhibit planktonic behavior offshore with antenna propulsion in mysis, and abdominal propulsion in megalopa. While the nauplii utilize yolk granules within their bodies. Feeding starts in protozoea and mysis (collectively called zoea) substages. The magalopa with the earlier juvenile stage (traditionally called postlarvae or “fry” for commercial purpose) is transparent with dark brown streak on the ventral side tip the antennular flagellum to the tip of the telson. Under laboratory conditions, postlarvae become benthic on the sixth day of the post-larval stage. In natural conditions, the megalopa enters the nursery ground. The carapace length of megalopa varies between 1.2 and 2.3 mm (Solis, 1988).

Juvenile. The earlier juvenile stage has transparent body with dark brown streak on the ventral side as in the megalopa. The earlier juvenile stages as follows: (1) relatively shorter sixth abdominal segment compared to the carapace length, (2) greater body size, (3) complete rostral spine formula, (4) complete gill system, and (5) benthic behavior (Solis, 1988).

In the later stage, the body becomes blackish in color bulky, and the rostrum has 7 dorsal and 3 ventral spines. The juvenile crawls using the pereopods and swims using the pleopods as in adults. The carapace length (CL) varies from 2.2 to 11.0 mm (Solis, 1988).

Adolescent. This stage resembles the adult prawn. Sexes are now distinct beginning at 11 mm CL. The minimum size of male possessing a jointed petasma is about 30 mm CL and minimum size of females possessing adultlike thelycum is about 37 mm CL. The carapace length of the adolescent varies between 11 and 34 mm (Solis, 1988).

Subadult. This stage is the onset of sexual maturity. The male possesses spermatozoa in its terminal ampoules. The thelycum of the female now contains spermatozoa. At this stage (30 mm CL), female grows faster and migration from nursery spawning grounds begins. In the course of migration, final copulation takes place between males and females having minimum of 37 mm and 47 mm CL respectively (Solis, 1988).

Adult. This stage has appendages very similar to the subadult and is characterized by completion of sexual maturity. It differs only with the subadult in size and habitat. Males possess spermatozoa, and females start to spawn offshore although a few spawn in shallow water. A second or more copulation may occur in majority of the species. Major habitat is the offshore area up to about 160 m depth. Carapace lengths of adults vary between 37 and 71 mm in males and 47 and 81 mm in females (Solis, 1988).

The diagram of the life history phases of *Penaeus monodon* shown in Figure 2.4

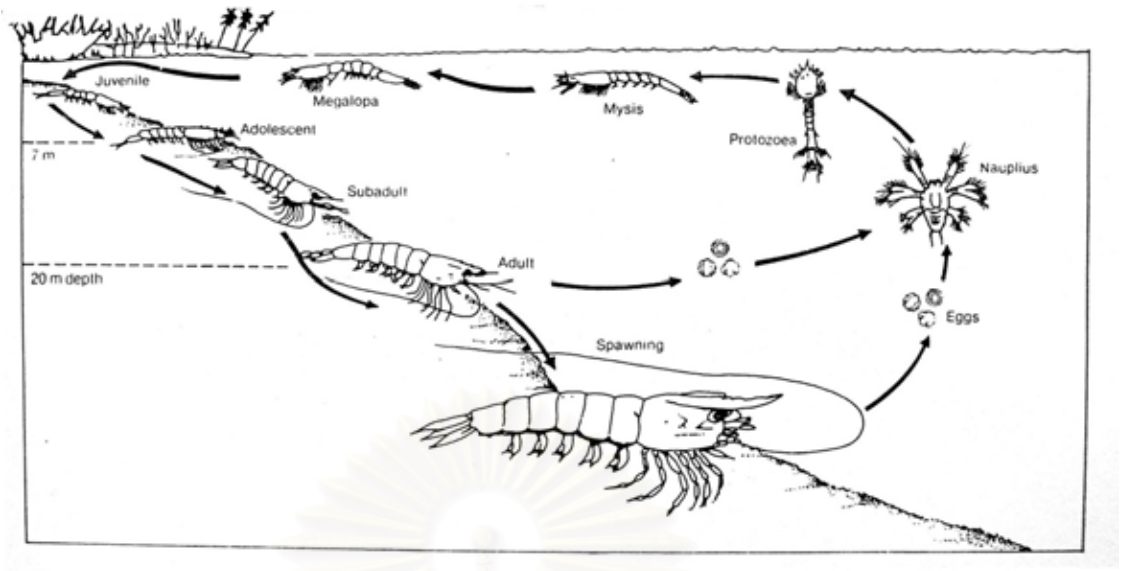


Figure 2.4 The diagram of the life history phases of *Penaeus monodon* (Solis,1988)

Longevity

There is no reliable method developed to determine the age of an individual prawn. It is believed that the life span of *P. monodon* is one to two years (Solis, 1988).

Molting, growth and maturation

The external shell (exoskeleton) of crustaceans is capable only limited expansion. Growth occurs through molting (shedding the exoskeleton or ecdysis) at intervals throughout the life. The rate of growth is a function of the frequency of molting and the increase in size at each molt. Adverse nutritional or environmental conditions can decrease both functions. The main sequence of the events in the cycle is: (1) Accumulation of mineral and organic reserves; (2) Removal of material from the old shell and formation of the new exoskeleton; (3) Ecdysis, accompanied by the uptake of water; (4) Molecular strengthening of the exoskeleton by rearrangement of organic matrices and deposition of inorganic salts and (5) Replacement of fluid by tissue growth.

The frequency of molting varies naturally between species, with size and with age. Young shrimp larvae molt two or three times in a day, juveniles every three to 25 days depending on temperature and species. Crustaceans often eat cast shells, a convenient source of minerals which would otherwise be lost. Mineralization of the new shell is affected by the availability of particular ions (calcium, bicarbonate) and pH) in the surrounding waters, in the diet, and in freshwater animals from materials stored in the body prior to molting. The changes that arise in water composition during intensive culture and particularly in recirculation system can have a major effect in the mineralization process and on the animal's ability to control blood pH.

Newly-molted individuals are particularly vulnerable to cannibalism especially under crowded culture conditions. Neither the presence of shelters nor the availability or adequate food eliminates cannibalism, although their absence may increase it. Attempts to reduce cannibalism by synchronizing molting or ameliorating aggressive behavior through claw removal or by giving drugs have met with little success.

Species grown in outdoor ponds may tend to molt in phase with the lunar cycle or in response to a change of water. Such effects are taken into account when the decision to harvest is made.

The molting cycle and sexual maturation are two vital physiological processes influenced by the X-organ sinus gland system situated in the crustacean eyestalks. Surgical removal of only one set of the glands from penaeid prawn that do not rapidly mature in captivity (unilateral eyestalk ablation) is sufficient to reduce the circulating gonad-inhibiting hormone to levels that permit rapid maturation (Lee and Wickins, 1992).

2.2 Crustacean endocrinology

Many behavioral and physiological processes in crustacean are regulated by endocrine system. These include circadian and tidal rhythmicity, locomotion, posturing, chromatic adaptation, carbohydrate and lipid metabolism, water and ionic balance, molting, growth, regeneration, gonadal development, reproductive physiology and cardiac activity (Beltz, 1988). The X-organ sinus gland system in the eyestalk is the major neuroendrocrine regulatory center in crustacean. The postcomissure organ at the posterior fusion of the circumesophageal connective receives axons from the brain and contains a number of chromatophorotropic peptide hormones. The pericardial organs, which is stretched across openings of gill veins into the pericardium, receives axons from neurosecretory cell bodies located in the ventral ganglion and also from other cell bodies, and releases hormone that affect the hear. The Y-organs are the source of molting hormone located in the maxillary or antennary segment. The androgenic gland, present only in male, affects the normal differentiation and muturation of the male reproductive system and the male's secondary sexual characteristics. This gland is found attached to the ejaculatory regions of the vas deferens. The ovaries appear to secrete hormones that control differentiation of the female secondary sex characteristics. The mandibular organ, which is generally located near more anterior to the Y-organ. Methyl farnesoate, a precursor for juvenile hormone, is one secretion of the mandibular gland (Beltz, 1988, Fingerman, 1997; Withers, 1992)

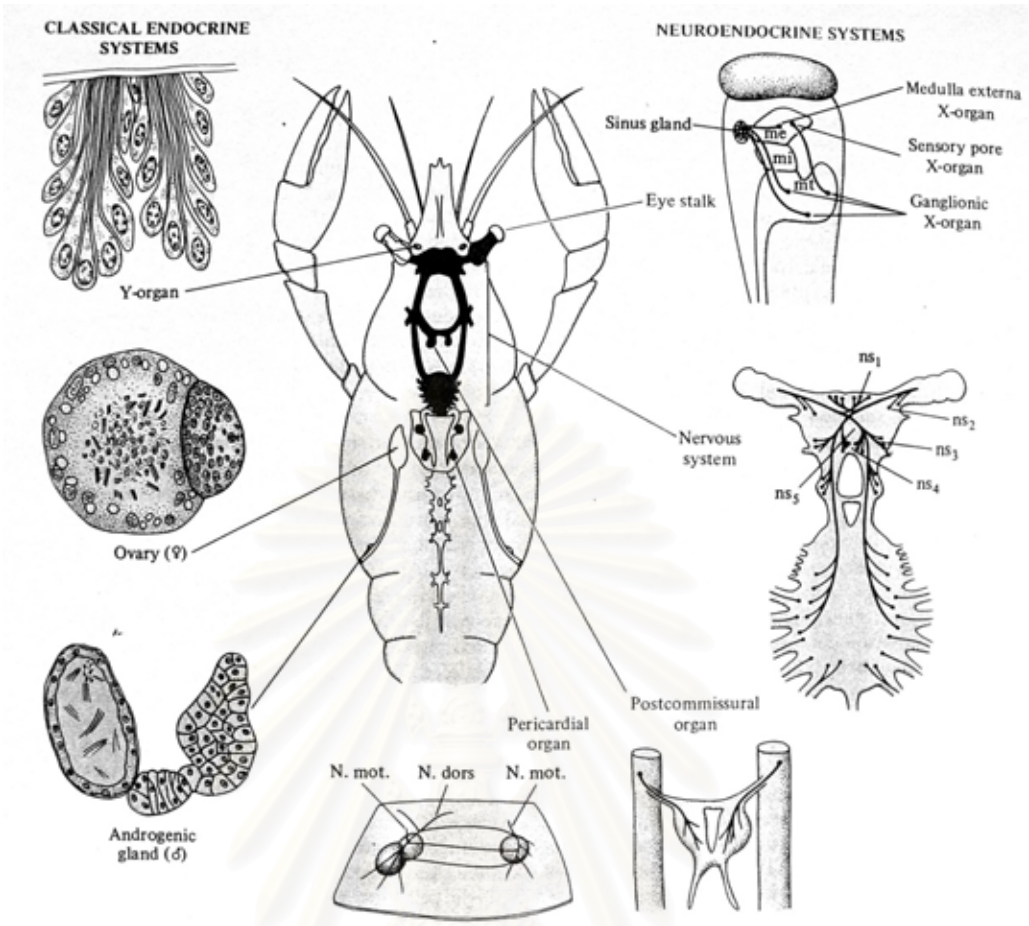


Figure 2.5 The generalized endocrine system of a crustacean. (Withers, 1992)

2.3 Crustacean Eyestalk

Eyestalk structure

The crustacean eyestalks are compound since they are made up of many photoreceptor units called ommatidia. The dioptric portion of the ommatidia is composed of (proceeding distally): the rhabdoms and rentinular cell bodies (Rha), the retinular cell nuclei (Ret), the cone cells (Con), the crystalline tracts (Crt), the crystalline cones (Crc), the cone cells (Con) and the outer lying cuticle (Cut) (Fig 2.6) (Bell and Lighter, 1997; Hickmam et al., 1993).

In a dissected exsoskeleton eyestalk, the internal eyestalk contains three ganglion : the medulla terminalis (MT), the medulla interna (MI) and the medulla

externa (ME). Between the ommatidia and the medulla externa lies the lamina ganglionaris (LG). The sinus gland is noted between the medulla externa and medulla interna. The X-organs are located in the several different places within the eyestalk ganglia such as medulla terminalis ganglionic X-organ, medulla externa ganglionic X-organ.

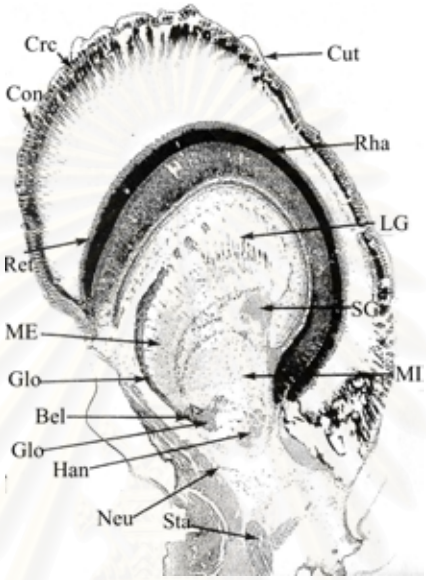


Figure 2.6 Overall view of a compound eye of Penaeid prawn (Bell and Lightner, 1997)

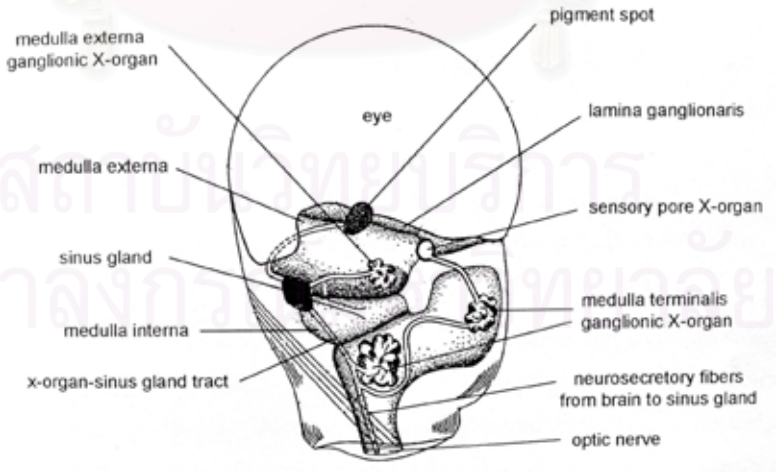


Figure 2.7 The eyestalk of shrimp *Leander serratus* (Carlisle and Knowles, 1959)

X-organ Sinus gland system

The X-organ sinus gland system, which is the major neuroendrocrine system in eyestalk crustacean, is the source of neuropeptide hormone which partake in the control of a wide range of physiological function such as chromatophore contol, inhibitory control of molting, carbohydrate metabolism, sexual differentiation. The structure and function of this system is analogous to the vertebrate hypothalamo-neurohypophyseal system as well as to the corpus cardiacum of insect (Beltz, 1988).

The X-organ is a cluster of neurosecretory cells located in the medulla terminalis (MT-XO). There are also neurosecretory cells in the medulla interna (MI-XO). Several neurohormones are produced in the X-organ and transported via an axon tract to the principle neurohemal organ, the sinus gland (SG), where they are stored and released into haemolymph (Keller, 1992).

The sinus gland as a neurohemal organ is a cluster of neurosecretory axonal terminals, with the axons going to it serving as pipeline for neurohormone produced in the cell bodies where these axons originate (Fingerman, 1997). In a freshly dissected eyestalk, the sinus gland is visualized as an iridescent white structure at the surface of optic ganglia on the dorsal or dorsal-lateral side in the region between the medulla interna and the mudulla externa (Sullivan, 1982).

2.4 Neuropeptide hormone in eyestalk

By means of chromatography, enzymatic digestion, microseqencing, mass spectrometry, bioassay, immunological technique and molecular cloning, various neuropeptides from the eyestalk have been identified.

2.4.1 *The CHH/MIH/GIH peptide family or The CHH family*

This family includes neuropepides (crustacean hyperglycemic hormone-CHH, molt-inhibiting hormone-MIH, gonad inhibiting hormone-GIH or vitellogenesis-inhibiting-VIH hormone and mandibullar inhibiting hormone) which are synthesized by neurosecretory cells in medulla terminalis X-organ (MT-XO) in the eyestalk of decapod crustacean (crab, shrimp, crayfish and lobster). The general stucture of this family consists of 72-78 amino acid residues in length and similar in amino acid sequence, and have molecular weights of 7,000-8,000 Da and have six conserved cysteine residues that form three disulfide bonds (Keller, 1992).

- Crustacean hyperglycemic hormone-CHH

The CHH, which involves on the regulation of carbohydrate metabolism, is the most abundant neuropeptide in the sinus gland. CHH has been isolated from several decapod crustaceans such as in the crab, *Carcenus maenas* (Kegel et al., 1989); in the lobster, *Homarus americanus* (Tensen et al., 1991); in the crayfish, *Procambarus bouviei* (Huberman et al., 1995), in the shrimp, *Penaeus japonicus* (Yang et al, 1997), and *Penaeus monodon* (Davey et al., 2000).

In 1989, Kegel et al. isolated and sequenced the first CHH of this family from the sinus gland of the shore crab, *Carcinus meanas* using HPLC. The determination of the amino acid sequence using DABITC-PITC double-coupling method revealed 72 amino acid residues. The molecular mass is 8,524 Da by fast atom bombardment-mass spectrometry (FAB-MS). Three disulfide bridge are present and both N- and C- terminal are blocked.

Kegel et al. (1991) found that the primary structure of the major form of CHH was isolated from sinus gland of the crayfish, *Orconectes limosus* using RP-HPLC and the sequence was detemined by manual Edman microsequencing. It is

a 72 amino acid residues with a molecular mass of 8,400 Da. In the number of residues, it is identical to the CHH of *Carcinus maenas* (61%) and very similar to MIH of *Homarus americanus* (81%). The CHH has the N-terminal as pGlu and C-terminal as Val-NH_2.

In the lobster, *Homarus americanus*, Tensen et al. (1991a, 1991b) isolated two sets of two isoforms of the CHH from sinus gland using a two-step HPLC purification. Characterization of the purified peptides revealed that all CHHs (CHH-I,-II, VI,VII) consisted of 72 residues, including six cysteines. Molecular mass of the CHH-I/II and VI/VII were 8,578 Da and 8,655+25 Da using FAB-mass spectrometry. The N-terminal of the partial amino acid sequences of all peptides are pyroglutamate. Subsequently, based on partial amino acid sequence, they reported the sequences of two CHHs (CHH-A and CHH-B) encoded from the eyestalk cDNA. The CHHs sequence and amino acid compositions were also fully agreed with results on the purification and subsequent chemical characterization.

Yang et al. (1995) extracted 1,500 sinus glands from the kuruma shrimp, *Peneaus japonicus* with 0.9% NaCl in 30% aqueous acetonitrile and purified by a single run of reverse-phase HPLC and found that five major peak materials from peak No. 34, 40, 42, 48 and 50. They were thought to be CHH family peptides, whose protonated molecular ionpeak were observed at m/z 8,368, 8,487, 8,353, 8,328 and 8,314 respectively. The structure analysis of the No.42 peptide consisted of 72 amino acid residues, that had an amidated carboxy-terminus and showed the hyperglycemic activity indicating that this peptide is a hyperglycemic hormone in this species. In 1997, Yang isolated and characterized six CHH family peptide from the sinus gland extracts of the same species using a single-step RP-HPLC, enzymatic digestion, FAB-

mass spectrometry and bioassay for hyperglycemic activity. Five of these peptides (Pej-SGP-I, -II, -III, _V and VI) expressed hyperglycemic activity. These consisted of 72 amino acid residues with a free amino terminus and amidated carboxy-terminus. The dose-response relationship of these peptides showed that they were equally potent but had different efficiencies, which were in the order of Pej-SGP-V, -VII > -III, -I > -II.

Gu et al. (1998, 2000) characterized the putative CHHs (CHH-A, -B) from a cDNA isolated from eyestalk of *Metapenaeus ensis*. These cDNA consisted of a signal peptide, a CHH- precursor-like peptde (CPRP) and the peptide. The sequence of the CHH-like-peptides (MeCHH A and MeCHH B) consisted of 74 amino acid residues and six cysteine were located at conserved position. Both of CHHs share 98% amino acid sequence identity. These peptides were express in the eyestalk of female and male but not in hepatopacreas, muscle, ovary, testis, nerve cord, epidermis, heart, and pre-hatch nauplius.

In the CHH of *Macrobrachium rosenbergii*, Sithigorngul et al. (1999) extracted 5,000 eyestalks with 90% methanol : 1% acetic acid : 9% water, purified by RP-HPLC and bioassayed a peptide with CHH activity, amino acid sequence was determined by automated microsequencing and analyzed for molecular mass by MALDI-TOF. The Mar-CHH composed of 71 amino acid residues and contained six residues of cysteine. The N-terminal is alanine and the C-terminal is amidated valine. However, the whole sequence in Mar-CHH seems to be incomplete since only 71 amino acids were identified and MALDI-TOF analysis indicated that one amino acid may be missing at position 71. Subsequently, Sithigorngul et al. (1999) found that the 71st residue of Mar-CHH is threonine using anti-T antibody

(made against YANAVQTV-NH_2). Therefore, it is quite likely that residue certain that Mar-CHH must consists of 72 amino acid residues with theonine at position 71.

In the atlantic ocean shrimp, *Penaeus(litopenaeus) schmitti*, Huberman et al. (2000) isolated and characterized the CHH from a crude extract of the sinus glands using a combination of RP-HPLC, Edman degradation, enzymatic digestion, TLC of densyl-amino acid, mass spectrometry, and bioassay. It consists of 72 amino acid residues that correspond to a mass of 8,359.4 Da. Its N-terminus is free and C-terminus is amidated.

Davey et al. (2000) identified and characterized five isoforms of CHH (Pm-sgp-I to –V) from *P. monodon* eyestalk cDNA. The encoded CHH peptides consisted of 72 amino acid long with molecular mass of 8511.05, 8322.94, 8349.90, 8294.99 and 8214.87 Da, respectively using ESI-FTMS. They have six conserved cysteine residues and are amidated at the carboxy terminus.

Furthermore, CHH in the *P. monodon*, Udomkit et al. (2004) reported another three CHH (Pem-CHH1, Pem-CHH2 and Pem-CHH3) peptides from *P. monodon* eyestalk cDNA. These consisted of 74 amino acid residues. Most of the differences in the amino acid sequence among the three types of PemCHH lie within the first five amino acid residues at the N-terminus of their mature peptide. 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*.

- Molt-inhibiting hormone-MIH

MIH plays a central role in the molting process as it is responsible for the inhibition of ecdysteroid systhesis in the Y-organ (de Kleijn and Van herp, 1998). This neuropeptide hormone has been reported in several species.

In 1994, Su cloned and sequenced a cDNA encoding a MIH-like neuropeptide from the *Penaeus vannamei*. This peptide consisted of a 72 amino acid residues and shared various degree of homology to other related peptides. By means of Northern hybridization, she showed that MIH-like peptide genes are expressed in the eyestalk but not in muscle.

Chung et al. (1996) isolated and characterized putative molt-inhibiting hormone from sinus glands of the edible crab, *Cancer pagurus* using HPLC, followed by fractional bioassay (inhibition of ecdysteriod synthesis by Y-organs), immunoassay (using antisera raised against *Carcinus* MIH) and automated Edman degradation of endroproteinase-derived fragments. This peptide is a 78 amino acid residues (Mw. 9,194) with free N- and C-termini and three intrachain disulfide bridges.

Nagasawa et al. (1996) isolated and sequenced a molt-inhibiting hormone (Prc-MIH) from the sinus gland of the American crayfish, *Procambarus clarkii* using RP-HPLC followed the purified Prc-MIH by bioassay for MIH. It comprised 75 amino acid residues and had amidated carboxyl terminus.

Aquilar et al. (1996), used RP-HPLC, bioassay for MIH activity, enzymatic digestion, manual Edman degradation and MALDI-TOF mass spectrometry to isolate and sequence MIH from the sinus gland of the Mexican

crayfish, *Procambarus bouvier* (Ortmann). It consisted of a 72 amino acid residues (Mw. 8,322 Da) with six cysteines residues forming three disulfide bridges. It has blocked N- and C- termini and lacks tryptophan, histidine, and methionine. MIH shows striking similarity to the CHH isomorphs of *Procambarus bouvier* (90% identity and to the MIH from *Homarus americanus* (79% identity) and *Penaeus vannamei* (46% identity). It is also related to the MIH from *Carcinus maenas* (28% identity) and *Callinectes sapidus* (28% identity).

Yang et al. (1997) isolated six major peptide (Pej-SGP-I-VI) that belong to the CHH family from the sinus gland extracts of the kuruma shrimp, *Penaeus jaoponicus*. They found that Pej-SGP-IV was active in inhibiting ecdysteroid synthesis by *in vitro* assay using the Y-organ of the crayfish, *Procambarus clarkii*. Pej-SGP-IV consisted of 77 amino acid residues with both free amino and carboxyl termini. Subsequently, Ohira et al. (1997) isolated a cDNA encoding a MIH (Pej-SGP-IV) and used the cDNA to study changes in the levels of the Pej-SGP-IV mRNA in the eyestalk during molting cycle. Level of mRNA did not decrease significantly at the premolt stages suggesting that the synthesis modification and/or secretion of MIH might be regulated post transcriptionally.

Gu et al. (1998, 2002) cloned and characterized two MIH-like neuropeptides (Mee-MIH-A and Mee-MIH-B) from the eyestalk cDNA library of *Metapenaeus ensis*. They consisted of 79 amino acid residues with six conserved cysteines . Mee-MIH-B showed 70% amino acid identity to the Mee-MIH-A.

Krungkasem et al. (2002) cloned two cDNA encoding MIH-like peptides (Pem-SGP-C1 and -C2) from the *P. monodon*. Both of the mature hormones consisted of 75 amino acid residues, which differed from one another at 2 positions:

Asp^{4} and val^{70} in Pem-SGP-C1 and Glu^{4} and Ile^{70} in Pem-SGP-C2. Using RT-PCR, it was demonstrated that both Pem-SGP-C1 and Pem-SGP-C2 transcripts were detected only in cDNA synthesized from total RNA isolated from the eyestalk but not in that from brain, thoracic ganglia, abdominal ganglia, abdominal muscle, hepatopancreas or heart tissue of *P. monodon*.

- Gonad-inhibiting hormone-GIH or Vitellogenesis-inhibiting hormone-VIH

The first investigator to provide direct proof for a hormone involved in reproduction was Panouse (1943) (cite from Fingerman, 1997). He showed that ablation of the eyestalks of *Palaemon serratus* results in rapid maturation of the ovaries. This effect is due to the fact that the sinus gland contains a gonad-inhibiting hormone (GIH). GIH is now known to be present in the sinus glands of males also. This technique of eyestalk ablation to induce gonadal maturation is now used in shrimp farms worldwide. In female GIH inhibits yolk synthesis, this hormone is sometimes called vitellogenesis-inhibiting hormone (VIH). The study of a GIH has been less successful than the study of CHH and MIH.

In1987, Soyez et al. isolated VIH with 7-8 kDa from the sinus glands of the lobster, *Homarus americanus* using RP-HPLC and sodium dodecyl sulfate urea polyacrylamide gel electrophoresis (SDS-Urea PAGE). This VIH was characterized using an *in vivo* heterologous bioassay developed in the bilateral eyestalk ablated female grass shrimp, *Palaemonetes varians* by detecting the inhibition of oocyte development. They also found that the complete amino acid sequence of VIH consisted of a 77 amino acid residues with a free N-terminus and three disulfide bridges. Its molecular mass was 9,135 Da using gas-phase

microsequencing and fast-atom bombardment mass spectrometry. The amino acid sequence related to CHH and MIH (Soyez et al., 1991).

The neuropeptide with vitellogenesis-inhibiting hormone (VIH) activity was isolated from a crude extract of sinus glands of the crayfish, *Procumbarus bouvieri* using a single step of RP-HPLC on a μBondapak-phenyl column, a heterologous *in vitro* bioassay as the depression of vitellogenin biosynthesis by cultured *Penaeus vannamei's* ovaries. It consisted of 72-74 amino acid residues with 8,388 $\pm$ 2 Da and a blocked N-teminus (Aquilar et al.,1992).

Edomi et al. (2002) have cloned and sequenced a cDNA encoding a GIH from the eyestalk of the Norway lobster, *Nephrops norvegicus*. The encoded GIH consisted of 81 amino acid residues. The amino acid identity of GIH with other crustacean MIHs and MOIH is of 41-56%, the identity with CHH is 27-33%, the highest amino acid identity (96%) is with *Homarus americanus* GIH.

- Mandibular-inhibiting hormone-MOIH

Mandibular organs (MO) produce a crustacean juvenile hormone, methyl farnesoate (MF). MF synthesis is inhibited by a neuropeptide from the X-organ sinus gland system in the eyestalk, called mandibular organ-inhibiting hormone (MOIH). MOIH is a member of the expanding CHH family.

Wainwright et al. (1996) isolated and characterized MOIH from sinus gland of the crab, *Carcer pagurus* by means of HPLC, peptide sequencing, mass spectrometry and mandibular organ bioassay. They found that two purified neuropeptide (MOIH-1 and –2) inhibited MF synthesis. The complete sequence of MOIH-1 consisted of 78 amino acid residues (MW. 9,235.6 Da) with unblock termini

and three intrachain disulfide bridges. MOIH-2 appeared to be almost identical to MOIH-1 with the exception of a Gln for Lys substitution at position 33. This sequence shows similarities (50-60%) to members of the CHH family.

Liu et al.(1997) isolated and sequenced MOIH from the sinus gland of the spider crab *Librinia emarginated*. It consisted of 72 amino acid residues (MW. 8,490.5 Da) with pyroglutamic acid at the N-terminus and NH_2 at the C-terminus. The activity showed that it inhibited MF synthesis *in vitro* and had hyperglycemic activity when it was injected into crabs.

2.4.2 *The Chromatophorotropins*

These are peptides classically known as pigmentary effector hormones in crustacean. Crustacean have four types of Choromatophores: erythrophores, melanophores, xanthophores, and leukophores. These cell are loaded with pigment granules that concentrate or disperse as ordered by specific chromatophorotopins (Huberman, 2002)

- Red pigment concentrating hormone (RPCH)

RPCH was first isolated and sequenced from the eyestalks of the *Pandalus borealis* (Fernlund and Josefsson, 1972). This is octapeptide: pELNFSPGW-NH_2. The sequence of RPCHs from the several crustacean such as *Cancer magister, Carcinus maenas, Orconectes limosus, Penaeus japonicus,* are thought to be identicle to *Pandalus* RPCH. This indicated that the structure of RPCH is highly conserved among crustacean species (Roa, 2001).

- Pigment dispersing hormone-PDH

In1976, Fernlund isolated and sequenced PDH, an octadecapeptide from eyestalks of *Pandalus borealis*. It was then known as a hormone inducing light-adapting movements of pigment in the crustacean compound eye and causing pigment dispersion in epidermal chromatophores (Keller, 1992). PDHs were isolated and sequenced from several crustacean species.

In crab, *Cancer maggister*, Kleinholz et al.(1986) enabled the purification of an octadecapeptide PDH from the powdered lyophilized eyestalks using separation procedures including liquid partition, gel filtration, ion-exchange chromatography, partial chromatography, and RP-HPLC and automated Edman degradation. The sequence of thepeptide is: NSELINSILGLPKVMNDA-NH_2.

By means of molecular cloning, Kleijn et al.(1993) cloned and sequenced cDNA encoding PDH in the eyestalk of the crayfish, *Orconectes limosus*. The structure of the preprohormone consisted of a signal peptide, a PDH pprecursor-related peptide (PPRP) and the highly conserved PDH peptide at the carboxy-terminal end. *In situ* hybridization in combination with immunocytochemistry revealed four cell cluster expressing PDH in the optic ganglia of the eyestalk. Three clusters stained both with the PDH cDNA probe and the PDH antiserum, however, the perikarya in the lamina ganglionalis (LG) only stained with the PDH antiserum.

Desmancelles-Carette et al.(1996) isolated and characterized three different cDNAs encoding PDH-precursor consisted of a putative 22 or 23 amino acid signal peptide, a 34 amino acid PDH precursor related peptide (PPRP) and the 18 amino acid mature PDH. One of the PDHs amino acid sequences differs by the presence of a Leu at the position 11 when comparing with two sequences.

Yang et al.(1999) purified chromatophorotropic neuropeptide hormone from an aqueous extract of the sinus glands of the kuruma shrimp, *Penaeus japonicus* by two step of RP-HPLC and determined amino acid sequence by enzymatic digestion. Two peptide (Pej-PDH-I and –II) showed PDH activity. These consisted of 18 amino acid residues with a free amino terminus and an amidated carboxyl-terminus. Three amino acid residues at positions 11, 14, and 16 differed between two PDHs.

2.4.3 *The other peptide*

- FMRF amide-like peptide (FLPs)

The search for FMRF-like peptide (FLPs) began when Price and Greenberg (1977) isolated and identified the tetrapeptide FMRF-NH_2 (FMRFamide) from the bivalve molluse, *Macrocallista nimbosa*. FLPs have been isolated from every invertebrate phylum, making the “FMRFamide family” probably the largest and most widely distributed assemblages of structurally similar peptides. In most case FLPs have been found in the neurons and their processes (Mercier et al., 2003). In crustacean, 19 FLPs have been identified and sequenced from pericardial organs of the lobter, *Homarus americanus* (Trimmer et al., 1987), the blue crab, *Callinectec sapidus* (Krajniak, 1991) and the crayfish, *Procambarus clarkii* (Mercier et al.,1993), from the stomatogastric nervous system of *Cancer borealis* (Weimann et al., 1993) and also from the eyestalk of the *Macrobrachium rosenbergii* and *P. monodon* (Sithigorngul et al., 1998, 2001, 2002). These peptides range from 7 to 10 amino acid in length. They all share a common C-terminal sequence, the tatrepeptide XLRFamide (with X standing for F or R).

In eyestalk, Sithigorngul et al. (1998, 2001) isolated eight FLPs from 5000 eyestalks of *Macrobrachium rorenbergii* extracted in methanol/ acetic acid/ water (90:1:9). The extract was purified by eight steps of RP-HPLC using four kinds of column (C18, C8, Cyano and Phenyl) and three solvent system: acetonitrile in trifluoroacetic acid, in heptafluoroacetic acid and in triethylammonium acetate during purification. FLPs in the fraction were determined by dot-ELISA using mixture of three monoclonal antibody, FM-23(made against FMRFamide), AF1-62(made against KNEFIRFamide) and AF2-32 (made against KHEYLRF). Seven of them are new sequences. Furthermore, seven new sequences of FLPs were isolated from 9,000 eyestalks of *P. monodon* using combination of three anti-FMRFamide-like peptide monoclonal antibodies for monitoring FLPs in the fractions during purification process by dot-ELISA (Sithigorngul et al. 2001).

- NPY/PP-like peptide

Pancreatic polypeptide (PP) is a 36 amino acid peptide hormone, synthesized and released from endocrine PP cell situated within pancreatic islets. This peptide was initially identified from chicken pancreas (Kimmel et al., 1975). PPs have been identified from various vertebrate and invertebrate species. In crustacean, four isoforms of PPs were isolated and sequenced from the eyestalk of *P. monodon* using anti-PP6 antiserum by immunocytochemical method and dot-ELISA for monitoring the peptide during purification. This is the first report of PPs in crustacean.

- Allatostatin-like peptide familly

Allatostatins (ASTs) are neuropeptides, originally identified in cockroaches based on their ability to inhibit juvenile hormone biosynthesis by corpora allata, a major endocrine organ in insect (Pratt et al., 1991; Woodhead et al., 1989). The synthesis and secretion of juvenile hormone play an important role in larval

development and in the maturation and reproductive activities of insect. JH in some insects is involved in stimulation of vitellogenesis and cyclic maturation of oocytes. The common AST peptide family is characterized by a carboxy-terminal sequence re showed Y/FXFGL-NH_2 where X is either a A, D, N, G or S (Bendena et al., 1999; Stay et al., 1994). In the cockroach *Diploptera punctata*, ASTs have been found to function not only as regulators of juvenile hormone biosynthesis but also as potent inhibitors of muscle contraction (Lange et al., 1995) as well as having interneuronal functions, neuromodulatory roles, myoendocrine roles and direct actions on biosynthetic pathways (Bendena et al., 1999). In decapod crustacean, AST have been reported.

Skiebe and Schneider (1994) studied the effects of four *Diploptera punctata* allatostatin peptides on the stomatogastric nervous of the crab, *Cancer borealis*. All of the peptides had similar actions on the activity of neurons involved in rhythmic movements of the pyloric region of the stomach, decreasing the frequency of the pyloric rhythm in a dose-dependent manner. *Diploloptera* allatostatin 3 (D-AST-3) was slightly more effective than the others. Whole-mount immunocytochemistry with anti-*Diploptera* allatostatin 1 antibodies demonstrated the presence of allatostatin-like peptides in the paired commissural ganglia, the unpaired oesophageal ganglion and the stomatogastric ganglion, and in their connecting and motor nerves. Dense processes were labelled in the stomatogastric ganglion, 12-19 cell bodies and neuropil staining were found in each commissural ganglion. Two cell bodies were stained in the oesophageal ganglion and two pairs of cell bodies. The gastropyloric receptor neurons were also stained in peripheral nerves.

In1997, Duve et al. isolated 20 neuropeptides belong to the allatostatin superfamilly from extracts of cerebral and thoracic ganglia of the shore crab *Carinus maenas*. They were purified by HPLC, monitored by radioimmunoassay and identified by mass spectrometry and amino acid sequencing. The allatostatins are characterized by a common C-terminal pentapeptide sequence –YXFGL-NH_2. Previously such peptides have only been reported from insects. In *C. maenas*, however, there are only two types: thirteen of the peptides having a post-tyrosyl Ala and the other seven, a post-tyrosyl Ser. The crab peptides include the shortest allatostatins so far identified (YAFGL-NH_2) and YSFGL-NH_2) as well as the longest, a 27-residue peptide. The total of 20 peptides exceeds the highest number of allatostatins found in any of the insect investigated so far (14 in *Periplaneta Americana*). Immunocytochemistry has provided clue to the functions of the allatostatins in crustaceans by showing their widespread presence in the central and stomatogastric nervous system.

In 1999, Skiebe demonstrated that allatostatin-like peptides are distributed in the stomatogastric nervous system (STNS) and the pericardial organ (PO) of four decapod crustacean species: the crab *Cancer pagurus*, the lobster *Homarus americanus*, and the crayfish *Cherax destructor* and *Procambarus clarkii* by using wholemount immunocytochemical techniques with the *Diploptera* AST antibodies and confocal microscopy. The pattern of immunostaining suggested that AST-like peptides function as both neurohormone and as neuromodulators in the STNS of decapod crustacea.

In 1999 (Dircksen et al.) neuropeptides immunoreactive to an antiserum against allatostatin I (=Dipstatin 7) of the cockroach *Diploptera punctata* have been

detected in the central and peripheral nervous system of the crayfish, *Orconectes limosus* by immunocytochemistry and a sensitive enzyme immunoassay. Abundant immunoreactivity occurs throughout the central nervous system in distinct interneurons and neurosecretory cells. The latter have terminal in well-known neurohemal organ, such as the sinus gland, the pericardial organ, and the perineural sheath of the ventral nerve cord. Nervous tissue extracts were separated by RP-HPLC and fractions were monitored in the enzyme immunoassay. Three of several immunopositive fractions have been purified and identified by mass spectroscopy and microsequencing as AGPYAFGL-NH_2, SAGPYAFGL-NH_2, and PRVYGFGL-NH_2. The first peptide is identified to carcinustatin 8 previously identified in the crab, *Carcinus maenas*. The others are novel and are designated orcostatin I and orcostatin II, respectively. All three peptides exert dramatic inhibitory effects on contractions of the crayfish hindgut. Carcinustatin 8 also inhibits induced contractions of the cockroach hindgut. Furthermore, this peptide reduces the cycle frequency of the pyloric rhythms generated by the stomatogastric nervous system of two decapod species *in vitro*. These crayfish allatostatin-like peptides are the first native crustacean peptides with demonstrated inhibitory actions on hindgut muscles and the pyloric rhythm of the stomatogastric.

Huybrechts et al. (2003) determined the peptidome of the brain and thoracic ganglion of the Jonah crab, *Cancer borealis* using mass spectrometric methods. Fractions obtained by HPLC were characterized using MALDI-TOM MS and ESI-Q-TOF MS/MS. In total, 28 peptides were identified within the molecular mass range 750-3000 Da and eight peptides were de novo sequence. Comparison of the molecular masses obtained with MALDI-TOF MS with the calculated molecular masses of known crustacean peptide revealed the presence of four different peptide families;

allatostatin, orcokinin, kinin and FMRFamide related peptide family. At least nine putative allatostatins are present, five of them being identical to those previously in *Carcinus maenas* and four identical to those identified in *Penaeus monodon*.

In *P. monodon* (Duve et al., 2002), more than 40 peptides belong to the –Y/FXFGL-NH2 allatostatin superfamilly have been isolated in eleven RP-HPLC steps and identified from the central nervous system (CNS). The peptides can be arranged in seven sub-groups according to the variable post-tryrosyl residue represented by Ala, Gly, Ser, Thr, Asn, Asp, and Glu. Two of the residues (Thr and Glu) have not been observed in this position previously known in either insects or crustaceans. Also reported for the fist time for allatostatins, two of the peptides are N-terminally blocked by a pyroglutamic acid residue. Immunohistochemiscal study of the CNS of the *P. monodon*, with the same antisera as used to monitor the purification, confirms the widespread nature and complexity of allatostatinergin neural pathways in arthropods. Thus, all neuromeres of the brain and all except one of the ventral cord ganglia, process allatostatin neurons and extensive areas of allatostatin-innervated neuropile. In addition to the cytological evidence, the allatostatin act as neurotransmitters, associated with tissue as varied as eyes and legs, their presence in neurohemal areas such as the sinus gland and the perineural sheath of the thoracic ganglia suggests a neuroendrocrine function.

Table 2.1 The crustacean hyperglycemic hormone (CHH) in decapod crustacean eyestalk

Species	Tissue	Method	Result	Reference
Carcinus maenas	Sinus gland	HPLC enzymatic digestion DABITC-PITC double coupling FAB MS	Cam-CHH 72 residues MW. 8524 Da	Kegel et al., 1989
Orconectes limosus	Sinus gland	RP-HPLC Enzymatic digestion DABITC-PITC double coupling	Orc-CHH 72 resiues MW 8400 blocked N- and C-termini	Kegel et al., 1991
Homarus americanus	Sinus gland	RP-HPLC Enzymatic digestion FAB MS PCR	Two Hoa-CHH MW 8578 MW 8655$\pm$25 Da	Tensen etal., 1991a and 1991b
Penaeus japonicus	Sinus gland	RP-HPLC Bioassay MALDI-TOF MS	Pej-CHH 72 residues MW 8353	Yang et al., 1995
Penaeus japonicus	Sinus gland	RP-HPLC Bioassay Enzymatic digestion FAB MS	Four Pej-CHH 72 residues	Yang et al., 1997
Metapenaeus ensis	Eyestalk	PCR	Mee-CHH-A, 74 residues	Gu et al., 1998

Table 2.1 continued

Species	Tissue	Method	Result	Reference
Macrobrachium rosenbergii	Eyestalk	RP-HPLC Bioassay Dot-ELISA Edman degradation MALDI-TOF MS	Mar-CHH 71 residues blocked C-terminus	Sithigorngul et al., 1999
Metapenaeus ensis	Eyestalk	PCR	Mee-CHH-B 74 residues	Gu et al., 2000
Penaeus schmitt	Sinus gland	RP-HPLC Bioassay Edman degradation	Pes-CHH 72 residues MW. 8359.4 Da	Huberman et al., 2000
Penaeus monodon	Eyestalk	PCR ESI-FTMS	Five Pm-CHH MW. 8200-8500	Davey et al., 2000
Penaeus monodon	Eyestalk	PCR	Three Pm-CHH 74 residues	Udomkit et al., 2004

Table 2.2 The molt-inhibiting hormone-MIH in decapod crustacean eyestalk

Species	Tissue	Method	Result	Reference
Penaeus vannamei	X-organ	PCR	Pev-MIH 72 residues	Sun et al., 1994
Cancer pagurus	Eyestalk	RT-HPLC Bioassay Immunoassay Edman degradation	Putative MIH 78 residues MW. 9194 Free N- and C-termini	Chung et al., 1996
Procambarus Clarkii	Sinus gland	RT-HPLC *In vitro* bioassay FAB MS	Prc-MIH 75 residues blocked C-terminus	Nagasawa et al., 1996
Procambarus bouvieri	Sinus gland	RT-HPLC Bioassay MALDI-TOF MS Edman degradation	Prb-MIH 72 residues MW. 8322 Da blocked N- and C-termini	Aquilar et al., 1996
Penaeus japonicus	Sinus gland	RP-HPLC Bioassay Enzymatic digestion FAB MS	Pej-IV-CHH 72 residues	Yang et al., 1997
Metapenaeus ensis	Eyestalk	PCR	Mee-MIH-A	Gu et al., 1998
Metapenaeus ensis	Eyestalk	PCR	Mee-MIH-B	Gu et al., 2002
Penaeus monodon	Eyestalk	PCR	Two Pm-MIH	Krungkasem et al., 2002

Table 2.3 The gonad-inhibiting hormone (GIH) or vitellogenesis-inhibiting hormone (VIH) in decapod crustacean eyestalk

Species	Tissue	Method	Result	Reference
Homarus americanus	Sinus gland	RP-HPLC Bioassay FAB-MS	Hoa-VIH MW. 7-8 kDa	Soyez et al., 1987
Homarus americanus	Sinus gland	RP-HPLC Bioassay Micro sequencing FAB-MS	Hoa-VIH 77 residues MW. 9135 Da	Soyez et al., 1991
Procambarus bouvieri	Sinus gland	RT-HPLC Bioassay	Prb-VIH 72-74 residues MW. 8388±2 Da	Aquilar et al., 1992
Nephrops norvegicus	Eyestalk	PCR	GIH 81 residues	Edomi et al., 2002

Table 2.4 The mandibular-inhibiting hormone (MOIH) in decapod crustacean eyestalk

Species	Tissue	Method	Result	Reference
Cancer pagurus	Eyestalk	HPLC Bioassay Edman degradation Mass spectrometry	Cap-MOIH-1, -2 78 residues MW. 9235.6 Da	Wainwright et al., 1996
Libinia emarginata	Sinus gland	HPLC Bioassay Edman degradation MALDI-TOF MS	MOIH P22 72 residues MW. 8490.5 Da blocked N- and C-termini	Lui et al., 1997

Table 2.5 The chromatophorotopins in decapod crustacean eyestalk

Species	Tissue	Method	Result	Reference
Pandalus borealis	Eyestalk	Gel fitration Cation exchange Electrophoresis Thin layer Bioassay	Pan-RPCH Pan-PDH	Fernlund and Josefesson, 1972 ; Fernlund, 1976
Cancer magister	Eyestalk	Liquid partion Gel filtration Ion-exchange Partitonchromatography RP-HPLC Edman degradation Bioassay	Cam-PDH	Kleinholz et al., 1986
Orconectes limosus	Medulla terminalis and medulla interna	PCR	Pev-PDH 1-3	Desmoucelles-Carete et al., 1996
Penaeus japonicus	Sinus gland	RP-HPCH Enzymatic digestion MALDI-TOF MS Bioassay	Pej-RPCH Pej-PDH I, -II	Yang et al., 1999

Table 2.6 The FMRFamide-like peptide and NPY/PP-like peptide in decapod crustacean eyestalk

Species	Tissue	Method	Result	Reference
Macrobrachium rosenbergii	Eyestalk	RP-HPLC Dot-ELISA MALDI-TOF MS Edman degration	Eight FLPs	Sithigorngul et al., 1998; 2001
Penaeus monodon	Eyestalk	RP-HPLC Dot-ELISA MALDI-TOF MS Edman degration	Seven FLPs	Sithigorngul et al., 2002
Penaeus monodon	Eyestalk	RP-HPLC Dot-ELISA MALDI-TOF MS Edman degration	Four PP-like peptide	Sithigorngul et al., 2002

Table 2.7 The allatostatin (AST) in decapod crustacean

Species	Tissue	Method	Result	Reference
Cancer borealis	Stomatogastric nervous system (STNS)	Electrophysiology immunocytochemistry	Inhibited pyloric rhythm	Skiebe and Schneider, 1994
Cacinus maenas	Cerebral and thoracic ganglia	RIA HPLC MALDI-MS sequencing immunocytochemistry	twenty AST	Duve et al., 1997
Cancer pagurus Homarus Americanus Cherax Destructer Procambarus clarki	stomatogastric nervous system (STNS) and pericardial organ (PO)	immunocytochemistry	distributed in STNS and PO	Skiebe, 1999
Orconectes limosus	Central nervous system	Immunocytochemistry HPLC MALDI-MS microsequencing	three AST	Dircksen et al., 1999
Penaeus monodon	Central nervous system	RIA HPLC MALDI-MS Microsequencing Immunocytochemistry	more than 40 AST	Duve et al., 2002
Cancer borealis	Brain and thoracic ganglion	HPLC MALDI-TOF MS ESI-Q-TOF MS/MS	nine AST	Huybrechts et al., 2003

CHAPTER III

Preliminary of allatostatin-like neuropeptides and their putative receptor in eyestalk of the black tiger prawn, *Penaeus monodon*

3.1 Introduction

Aquaculture of the tiger prawn, *Penaeus monodon* is an industry of increasing importance to the economy of Thailand and other countries. Continued operation at optimal levels and further expansion of this industry has faced various challenges . One of the major limitations is the inability to produce adult broodstock in culture. This is attributable to a lack of information on crucial aspects of the biological basis of prawn growth, development and reproduction. In arthropods, growth, development and reproduction are dependent on hormones produced by the endocrine system. The crustacean eyestalk is an important neuroendocrine organ complex, comparable to the pituitary gland of vertebrates, and one of the major sources of hormones. Of these eyestalk hormonal regulators, the most extensively studied are peptides of the crustacean hyperglycemic hormone (CHH) family (Keller, 1992). These neuropeptides share sequence similarity and include CHH (Kegel et al., 1989), molt-inhibiting hormone (MIH) (Webster, 1991), vitellogenesis-inhibiting hormone (VIH) (Soyez et al., 1991) and mandibular organ-inhibiting hormone (MOIH) Wainwright et al., 1996). However, other neuropeptides purified from crustacean eyestalks include red pigment concentrating hormone (RPCH) (Fernlund and Josefsson, 1972), pigment dispersing hormone (Furnlund, 1976) and extended FMRFamide-related peptides (Sithigorngul et al., 1998, 2001).

Removal of the crustacean eyestalk is often required in captive adult broodstock of *P. monodon* to stimulate ovarian maturation. This suggests that an

inhibitory compound is being expressed in eyestalks. Extrapolating from studies on other crustacean species, one potential target of this inhibition is methyl farnesoate (MF) biosynthesis and/or secretion by the mandibular organ (Borst et al., 2001; Huberman, 2000). Hemolymph levels of MF increase upon eyestalk ablation. MF biosynthesis has been correlated with ovarian maturation and alterations in the morphological appearance of ovarian follicles (Laufer et al., 1998). Several neuropeptides have been implicated as MOIH's that may regulate MF biosynthesis. However, differing results have been obtained depending on whether an *in vitro* (Wainwright et al., 1996) or *in vivo* bioassay (Borst et al., 2001) was used to measure MF levels.

MF is the immediate precursor of juvenile hormone(JH) in insects whose biosynthesis occurs in the corpora allata. JH, in some insect species, is also under negative regulation by neuropeptides known as allatostatins. Allatostatins (ASTs) are neuroendocrine peptides, originally identified in cockroaches based on their ability to inhibit juvenile hormone biosynthesis by corpora allata (Pratt et al., 1991; Woodhead et al., 1989). The synthesis and secretion of juvenile hormone play and importance role in larval development and in the maturation and reproductive activities of insect. JH in some insects is involved in stimulation of vitellogenesis and cyclic maturation of oocytes. The cockroach AST family of peptides is characterized by a carboxy-terminal sequence Y/FXFGL-NH_2 where X is either a A, D, N, G or S (Bendena et al., 1999; Stay et al., 1994). In the cockroach *Diploptera punctata*, ASTs have been found to function not only as regulators of juvenile hormone biosynthesis but also as potent inhibitors of muscle contraction (Lange et al., 1995) as well as having

interneuronal functions, neuromodulatory roles, myoendocrine roles and direct actions on biosynthetic pathways (Bendena et al., 1999).

Immunohistochemical studies have localized AST antigenic determinants within the crustacean central nervous system including stomatogastric and peripheral nervous systems. In crustaceans ASTs may exert several functions that range from inhibiting pyloric rhythm (Skiebe and Schneider, 1994) to decreasing the gain of stomach muscle neuromuscular transmission (Jorge-Revera and Marder, 1997). ASTs also reduce evoked transmitter release from excitatory neuromuscular terminals presynaptically and then to decrease the contraction of muscle postsynaptically (Jorge-Revera and Marder, 1997). ASTs have also been immunolocalized to a pair of neurosecretory organs known as the pericardial organs that surround the crustacean heart. Through the pericardial organs, ASTs may be released into the hemolymph to serve a neuromodulatory function. Crustacean peptides with sequences that conform to the cockroach-type of AST have been purified from *Carcinus maenas* (Duve et al., 1997) *Orconectes limosus* (Dircksen et al., 1999) and recently from *P. monodon* (Duve et al., 2002).

In this chapter, we used immunohistochemical method on preliminary study of ASTs and putative recepteor to demonstrate that ASTs are distribute within eyestalk of *Pemaeus monodon* and likely to have roles as both neurotransmitters and neuromodulators.

3.2 Materials and methods

3.2.1 Animal

Juvenile (30-40g) and adult (80-100g) *Penaeus monodon* were obtained from farm nearby Bangkok, Thailand.

3.2.2 immunohistochemiscal localization of eyestalk peptides and proteins

3.2.2.1 *Preparation of paraffin section*

The eyestalks were removed from live juvenile and adult *P. monodon* and dissected the external shell then fixed in Bouin's fixative containing 0.5% glutaraldehyde immediately overnight. The fixed eyestalks were removed from fixative, dehydrated in a graded ethanol series, cleared in xylene and then embedded in paraffin by following : washed in tap water several times, soaked in 70%-90% ethanol for 2 hr each, in 95% for 24 hr, in 95%:butanol for 30 min, in butanol for 1 hr, in butanol : xylene for 30 min, in xylene twice times for 1 hr each, in xylene : paraffin for 30 min, in paraffin three times for 1 hr each and then embedded in block.

Serial eyestalk sections (8 or 50 μm thickness) were cut from paraffin-embedded block on a microtome, mounted onto gelatin-coated slides and baked for a day at 50^{o}C prior to deparaffinization. The deparaffinization and rehydration of the eyestalk sections were followed by soaking in xylene 3 times , xylene : butanol, butanol, 95% ethanol, 90% ethanol, 70% ethanol and water 3 times for 5 min each, respectively. Sections were blocked in P1+ (10% calf serum in PBS) for 30 min before use.

3.2.2.2 *Immunohistochemistry*

Serial sections (5 μm thickness) were prepared and processed for indirect immunoperoxidase staining. Rabbit polyclonal antisera used were specific for cockroach *Diploptera. punctata* ASTs: AST1 (=Dippu AST7 APSGAQRLYGFGL-NH_2), AST5 (DRLYSFGL-NH_2) and AST11 (YPQEHRFSFGL-NH_2) (Lange et al., 1995) and an AST-like peptide purified from *Calliphora vomitoria* CAST5 (GPPYDFGM-NH_2) (Duve et al., 1993). A rabbit polyclonal antiserum was raised against the peptide sequence NATRNEENITC derived from an amino-terminal fragment of the *Drosophila melanogaster* AST receptor (Dar-2) (Lenz et al.,2000; 2001). Each ASTs antiserum at the dilution of 1:3000 and the Dar-2 antiserum at the dilution of 1:1500 was incubated with eyestalk sections for overnight at 4^{o}C. A minimum of 10 sectioned eyestalks was used for each antiserum. After washing in PBS (15 min, 4 times), sections were incubated in secondary antibody (goat anti rabbit IgG H and L horseradish peroxidase conjugate ; GAR-HRP) dilute to 1:1000 for 4 hr at 37^{o}C or overnight at 4^{o}C then washed in PBS (15 min, 4 times). Finally slide were developed in a substrate solution containing 0.03% 3,3'-diaminobenzidine 4 HCl (DAB), 0.006% hydrogenperoxide (H_2O_2) in PBS. The immunoreactive sections were counterstained with eosinY, dehydrated in a graded ethanol series, cleared in xylene and mouted in permount. Immunoreaction were visualized as brown coloration against pink background of eosinY.

3.2.3 Specificity testing for the AST antisera

AST1, AST5 and CAST5 antisera were tested for specificity by antiserum adsorption to BSA-peptide conjugates. The peptide-specific conjugates were prepared by incubating 0.5 mg of each peptide with 10 mg BSA in PBS containing 0.5%

glutaraldehyde for 6 hr at room temperature. The conjugates were then dialyzed against four changes of PBS over 72 hr. Peptide conjugates were adjusted to a concentration of 5 mg/ml. BSA-glycine was prepared in the same manner. Antisera to AST1, AST5 or CAST5 were incubated overnight with their respective BSA-peptide conjugate or BSA-glycine (2 mg protein/ml). Samples were centrifuged at 10,000 X g for 10 minutes before alternate section application

3.2.4 Western blotting

3.2.4.1 Sample preparation

Eyestalk and *Drosophila* adults proteins were extracted by homogenization of 50 cuticle-free eyestalks in 0.5M Tris-HCl, pH 6.8, 0.1M NaCl, 0.1% Tween-20 and 1 mM phenylmethanesulfonyl fluoride. Homogenates were centrifuged at 14,500 X g for 30 min and the supernatant protein quantitated using the Bradford assay. The protein samples were mixed 1:1 with 2x treatment buffer boiled in 100 ^{o}C for 90 sec before electrophored

3.2.4.2 SDS-polyacrylamide gel electrophoresis

The slab gel consist of a 4% acrylamide stacking gel and 10% SDS-polyacrylamide gel. Protein samples (10 μg) were applied on stacking gel and electrophoresis was performed in 0.025 M tris-glycine, 0.1%SDS, pH8.3 tank buffer and running at constant voltage of 100V. Stop running when the dye marker moved to the bottom edge of separating gel.

3.2.4.3 Western blotting

After protein sample were separated on a 10% SDS-polyacrylamide gel. The gel was then transferred to a nitrocellulose membrane in Towbin buffer (3 hr at

50V, 4°C). Membranes were blocked with 5% Blotto (5% nonfat powdered milk, 0.1% Triton X-100 and 0.01% thimerosal in PBS) for 10 min at room temperature. Dar-2 antiserum (diluted 1:1,500 or 1:3,000 in 5% Blotto) was then added and incubated overnight at 4°C. Membranes were washed in 0.5% Blotto (15 min, 4 times) and incubated with GAR-HRP diluted 1:1000 in 5% Blotto for 8 hr at 4°C. Membranes were washed as described above and then developed in substrate solution (0.006% H_2O_2, 0.03% 3,3' diaminobenzidine 4HCl and 0.05% $CoCl_2$ in PBS) for 3-5 minutes.

3.3 Results

3.3.1 Immunohistochemical detection of AST

Antiserum specific to individual ASTs were used to detect cells in *P. monodon* eyestalks. Depending on the specific antiserum tested, cell bodies and neuronal processes were detected in most of the major areas defined in the eyestalk. Immunohistochemical staining with AST5 antiserum (Fig. 3.1A) detected the largest number of cell bodies and neuronal processes. With AST5, strong immunoreactivity was observed in approximately 3000 neuronal (5-7 µm diameter) cell bodies in the lamina ganglionalis. These cells have short processes that appear to extend to the rhabdome. Antiserum specific to AST1 (Fig. 3.1B), AST11 and CAST5 did not detect cells within the lamina ganglionalis (Table 3.1). The central nervous system of crustaceans extends into the eyestalks as ganglia known as the medulla externa (ME), medulla interna (MI) and medulla terminalis (MT) (Bell and lightner, 1988). All AST antisera tested identified neuronal processes that extend through the ME but did not detect defined cells within the interior of this region (Fig. 1A, B, 2A, C and 4A). AST antisera were strongly immunoreactive with medium (15 µm diameter) and large (30-

50 µm) cells located on the posterior side of the ME (Fig. 2A, 4A). None of the antisera detected cells or processes in the MI but AST5 and CAST5 antisera identified large nerve bundles that ran from anterior to posterior in the region between the MI and the MT (not shown). Immunoreactivity with antiserum to AST5, AST11 or CAST5 was concentrated in nerve terminals of the sinus gland (Fig. 3.1A and 3.4A). All AST antisera identified cells in the X organ located slightly ventral to the posterior end of the ME (Fig. 3.1A, 3.1B). The cell numbers detected within the X-organ vary dramatically (Table 3.1). Variation was found between individual eyestalks of shrimp of the same age using any given antiserum. Each antiserum also yielded differences in the average number of cells detected. The MT had immunoreactive cell bodies on both the anterior and posterior sides and numerous neuronal fibers extending throughout. Some neurons were found to send their processes to form large nerve bundles that extended down the posterior surface of the MT. Some nerve fibers from the MT appeared to extend through the optic nerve en route to the brain.

The specificity of the immunostaining was tested for AST1, AST5 and CAST5 by incubating each antiserum with either BSA coupled to glycine or BSA coupled to the peptide to which the antiserum was generated. Alternate eyestalk sections received either the BSA-glycine or BSA-AST pre-absorbed antiserum. In each case, BSA-AST completely blocked the immunohistochemical staining of specific neuronal cell bodies, nerve bundles and nerve fibers. Figure 2 shows a representative pre-incubation of CAST 5 antiserum with either glycine-BSA (Fig. 3.2A,C) or CAST5 coupled to BSA (Fig. 3.2B and D)

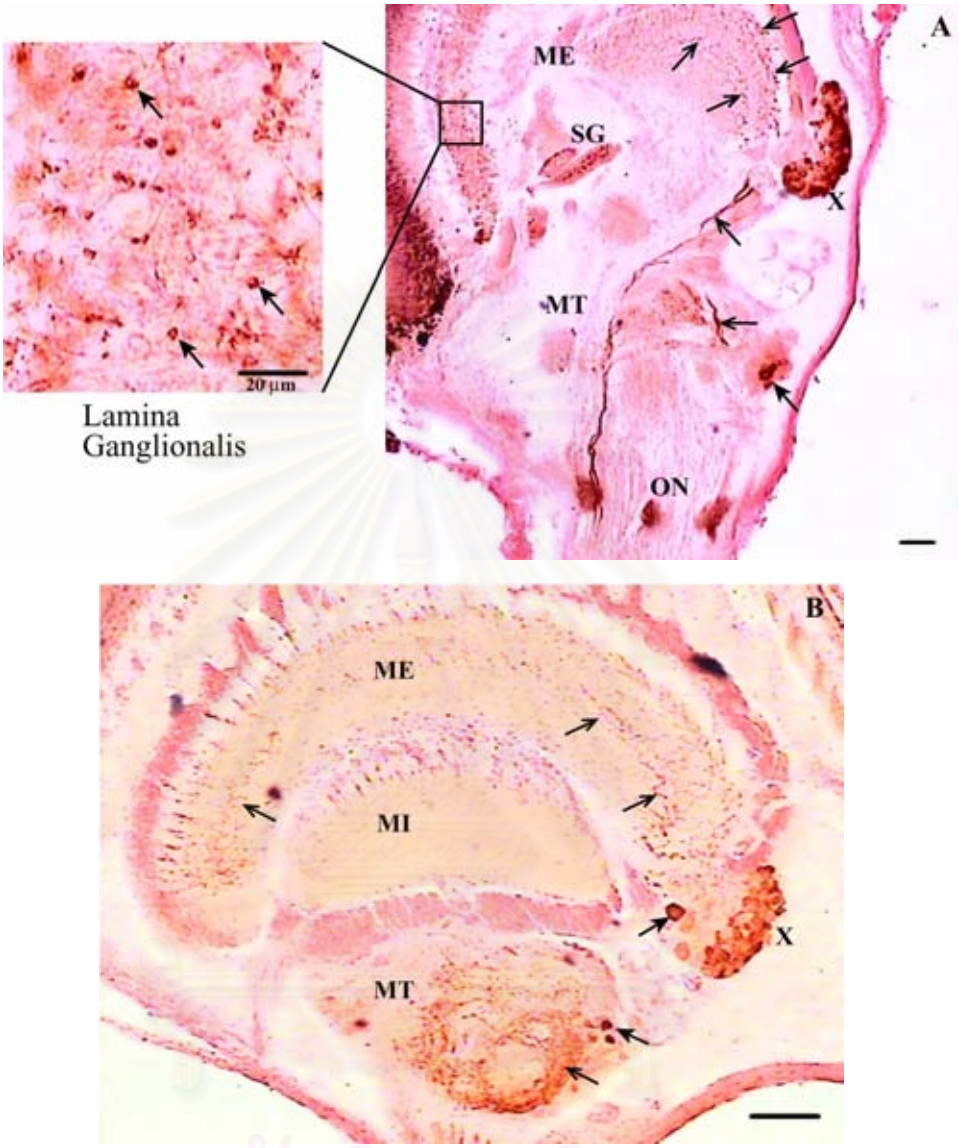


Figure 3.1 Immunolocalization of allatostatins using AST5 antiserum (Panel A) or AST1 antiserum (Panel B) on 50 µm sections of a *P.monodon* eyestalk. Staining of nerve fibers is evident in the lamina ganglionalis, the medulla externa (ME) and medulla terminalis (MT). No immunoreactive staining is evident in the medulla interna (MI). Numerous cells of the MT and the X-organ (X) display AST immunoreactivity. Nerve terminals of the sinus gland (SG) also display AST immunoreactivity. Open arrows indicate immunoreactive nerve fibers. Closed arrows indicate immunoreactive cell bodies Large immunoreactive nerve fibers can be traced through the optic nerve (ON). Scale bar = 100 µm.

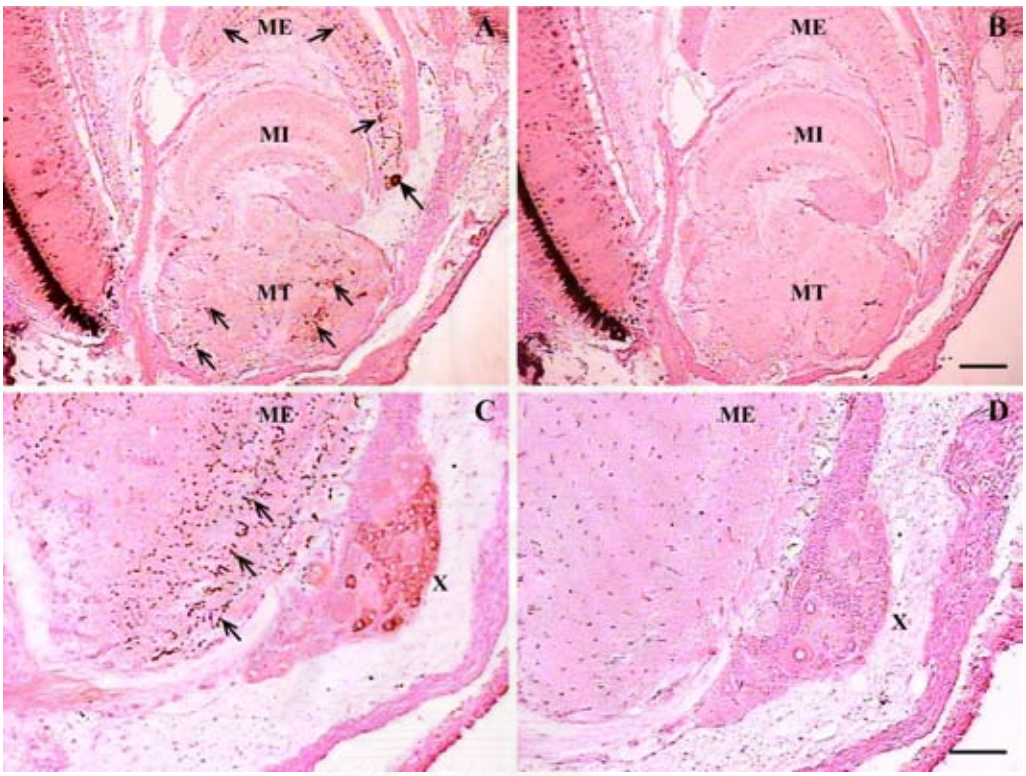


Figure 3.2 Localization of AST-like immunoreactivity using CAST5 antiserum on sections of a *P. monodon* eyestalk. Panels A and B are consecutive 8 µm sections. In Panel A, the antiserum was preabsorbed with BSA-glycine prior to tissue application. In Panel B the antiserum was preabsorbed with BSA conjugated to CAST5 prior to tissue application. CAST5 immunoreactive nerve processes are indicated with open arrows. A large (30-50 µm) immunoreactive cell ipsilateral to the ME is indicated with a closed arrow. X represents cells of the X organ in Panels C and D. Abbreviations are as defined in legend to Figure 3.1. Scale bar = 100 µm

Table 3.1 Summary of the number of cell bodies (Mean ±SD) or immunohisto-chemical localization detected in *P. monodon* eyestalks using different antisera

Antiserum raised against:	AST 1	AST 5	AST 11	CAST 5	Dar-2 A ♀ A ♂ J ♀ J ♂
LOCATION Lamina ganglionalis	-	3046±267*	-	-	- - - -
Ipsilateral to medulla externa including X organ	204 ± 42	168 ± 93	193 ± 84	90 ± 22	- - - -
Medulla terminalis	22 ± 12	7 ±6	21 ± 8	10 ± 12	- - - +
Sinus Gland	-	+	+	+	+ + + +

* Numbers based on counting cells from a minimum of 10 sectioned eyestalks.

3.3.2 Detection of a putative P. monodon AST receptor

Antiserum specific to a NH_2-terminal peptide fragment derived from the Dar-2 (Lenz et al., 2000 and 2001) recognized a single specific protein band in a *P. monodon* eyestalk protein extract separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose (Fig. 3.3, lane A). The antiserum did not recognize any protein from muscle protein extract of *P. monodon* (Fig. 3, lane B). A protein of approximately the same molecular size as detected in *P. monodon* eyestalk extracts was also detected in separated protein extract of adult *D. melanogaster* (Fig. 3.3, lane C).

Immunohistochemical staining of juvenile male and female *P. monodon* eyestalk sections with Dar-2 antiserum was most prominent in the sinus gland (Fig. 4B). Immunohistochemical staining of alternate 8 µm sections with either AST11 or

Dar-2 antisera showed that ASTs and the putative AST receptor share the same temporal expression in the sinus gland. Within the sinus gland, AST and Dar-2 immunoreactivity appeared concentrated to some nerve terminals (compare Fig. 3.4A and B). Detection of the putative *P. monodon* AST receptor was not evident in the lamina ganglionalis, ME or MI. In juvenile male eyestalks, detection of the putative AST receptor was found in cells of the MT X-organ (Fig. 3.4C, Table 3.1). Strong staining in the MT was evident in only a limited number of cells of the X-organ, with most sections showing weaker but consistent staining of a cluster of X-organ cells (Fig. 3.4C). Immunolocalization of putative AST receptor to the MT was not found in juvenile female *P. monodon* eyestalk sections. In adult (broodstock) *P. monodon* eyestalk sections, the Dar-2 antiserum showed immunostaining only in the sinus gland (Fig. 3.4D). No staining was found in the MT as was detected in juvenile male prawn.

Figure 3.3 Proteins extracts from *Drosophila melanogaster* adults (lane A), *P. monodon* muscle (lane B) or *P. monodon* eyestalk (lane C) were separated on a 10% SDS-polyacrylamide gel and then Western blotted and probed with antiserum prepared to the *Drosophila* allatostatin receptor Dar-2.

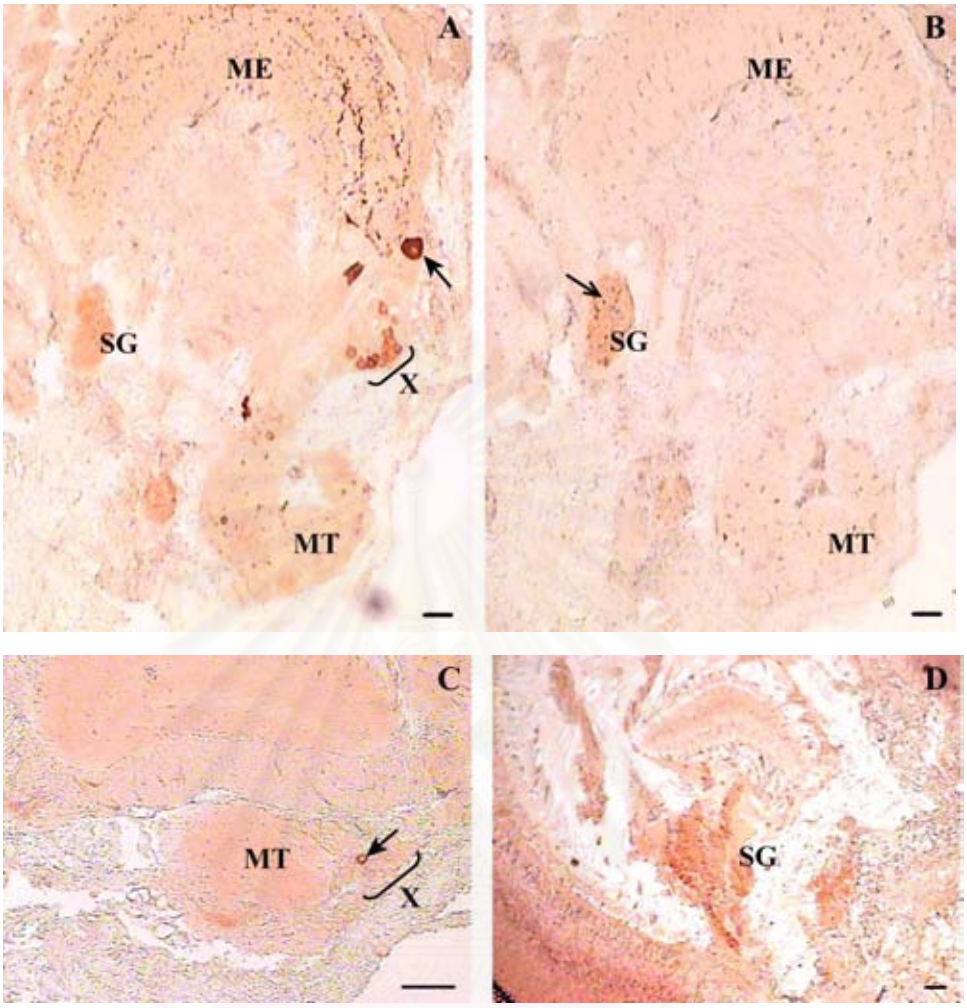


Figure 3.4. Immunohistochemical staining of 8 µm sections of *P. monodon* eyestalks. Panels A and B are consecutive sections incubated with either AST11 antiserum (Panel A) or Dar-2 antiserum (Panel B). In Panel A, a solid arrow indicates a large immunoreactive cell ipsilateral to the ME. In Panel B, an open arrow shows a representative immunoreactive nerve terminal within the SG. Staining of a juvenile male eyestalk section (Panel C) with Dar-2 antiserum detects a strong immunoreactive cell of the MT X-organ. In adult (broodstock) eyestalks immunostaining with Dar-2 antiserum is only detected in the sinus gland. Sinus gland (SG), medulla terminalis (MT). The region encompassed by cells of the X-organ (X) are indicated with a bracket in Panels A and C. Scale bar = 100 µm.

3.4 Discussion

AST immunoreactivity was detected in two ganglia, the ME and MT. In the ME, strong staining of nerve fibers was consistently found with all AST antisera tested. Detection of AST in ME nerve fibers appears to be specific, as immunostaining could be blocked by incubation of the antisera with the native peptides. ASTs were also localized to large (50 µm) cell bodies ipsilateral to the ME. These cell bodies did not appear to extend neuronal processes. AST immunoreactive nerve fibers and nerve bundles appear to occur throughout the MT. These observations are consistent with the ASTs acting as neurotransmitters or neuromodulators. These findings differ from a study that found no clearly defined fiber networks in the ME, MI or MT of *P. monodon* (Duve et al., 2002). The distribution of AST immunoreactivity also differed from that of the crayfish where all three ganglia showed strong AST immunoreactive nerve fibers (Dircksen et al., 1999). Certain AST sequences were also localized to the lamina ganglionalis that may suggest that AST-like peptides may function in the visual processing system. Large AST immunoreactive nerve fibers were also found traveling through the optic nerve, suggesting that ASTs may act as interneuronal transmitters, relaying information between the brain and the ganglia of the eyestalk. Immunolocalization of ASTs in the lamina ganglionalis of the insect visual system has recently been observed (Sarkar, Te Brugge and Orchard, personal communication). Associated with the MT is a cluster of neurosecretory cells known as the X-organ. Strong AST immunoreactivity was detected in hundreds of cells of the X-organ. The number of immunoreactive cells detected varied with the AST antiserum used. The X-organ cells project axons to the sinus gland which functions as a neurohemal organ (Andrew et al.,1978). AST immunoreactive nerve terminals/neurosecretory granules were found throughout the

sinus gland, suggesting that ASTs are released to the hemolymph and may function as circulating hormones through this route.

Antiserum for the Dar-2 appeared specific on Western blots, detecting only a single protein band of the same molecular size in both *Drosophila* and *P. monodon* eyestalk protein extracts, but did not detect any IR protein in a *P. monodon* muscle extract. The primary sequence of this receptor is 357 amino acids which would encode a 40,646 dalton protein. The receptor is likely to be a glycoprotein and PROSITE (Sigrist et al., 2002) detects 5 glycosylation sites of high probability. This might explain the retarded mobility of the protein on SDS-polyacrylamide gels. Our finding that the putative AST receptor is also localized to the sinus gland in both juvenile and adult *P. monodon* eyestalks suggests that ASTs may function to modulate sinus gland function, either providing a sensory feedback to the X-organ or possibly modulating sinus gland release of ASTs or other regulatory neuropeptides to the hemolymph. The X-organ-sinus gland system regulates through the action of numerous neuropeptides many physiological processes including carbohydrate metabolism, osmoregulation, molting, growth and reproduction (Huberman, 2000; Keller, 1992). Eyestalk ablation experiments have suggested that several neuropeptides controlling some of these vital processes are inhibitory regulators. For example MF produced by the mandibular organ is a potential candidate as a regulator of ovarian maturation and reproduction. Eyestalk ablation experiments have suggested that MF biosynthesis is under the negative control of two eyestalk neuropeptide MOIHs which have sequence similarity to MIH (Wainwright et al., 1996) based on *in vitro* bioassays. Recent data using a different bioassay suggests that these MIH–related MOIHs are not active in vivo (Borstvet al., 2001). As ASTs were discovered

based on their ability to inhibit JH production in insects, it is tempting to speculate that the ASTs, acting in conjunction with other neuropeptides, may regulate reproduction and/or molting in crustacea. Preliminary assays have suggested that ASTs have an effect on MF biosynthesis by crayfish mandibular organs *in vitro* (Tobe, SS, personal communication).

The significance of the sex-specific difference of the putative AST receptor expression in juveniles is unclear. It may be that the expression of the receptor in the MT X-organ cells in males is related to actions in the sinus gland. Alternatively, the putative AST receptor expression in the MT in males may activate signal transduction pathways that set up different states of gene expression in adult males versus females.

CHAPTER IV

Production of monoclonal antibodies against sinus gland neuropeptide in black tiger prawn *P. monodon*

4.1 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 (Keller, 1992, reviewed). Red pigment concentrating hormone (RPCH) was the first hormone isolated and sequenced from the eyestalk of *Pandarus borealis* (Fernlund and Josefsson, 1972), followed by pigment dispersing hormone (PDH; Fernlund, 1976). In crab *Carcinus maenas*, larger peptide hormones, crustacean hyperglycemic hormone (CHH) (Kegel et al., 1989), molt-inhibiting hormone (MIH) (Webster, 1991) and vitellogenesis inhibiting hormone (VIH) was identified in lobster *Homarus americanus* (Soyez et al., 1991) were identified. 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 family (Keller, 1992). 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 (Wainwright et al., 1996) and a CMG peptide which is structurally similar to a CHH identified in *Penaeus monodon* (Udomkit et al., 2000). With the development of automated microsequencing and molecular cloning, many peptides in this family have been identified in various species (Lacombe, 1999). Recently, eight isoforms of

FMRFamide-like peptide family were identified in *Macrobrachium rosenbergii* (Sithigorngul et al., 1998, 2001). 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 (Davey et al., 2000), CMG peptide (Udomkit et al., 2000), MIH (Krungkasem et al., 2002) 7 isoforms of FMRFamide (Sithigorngul et al., 2002a) and 4 isoforms of NPY/PP (Sithigorngul et al., 2002b). 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 (Duve et al., 2002) and the allatostatin-like peptide was immunohistochemically located in the eyestalk as well (Panchan et al., 2003). 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 (Sithigorngul et al., 2003). 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 (Sithigorngul et al., 1991) 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.

4.2 Materials and methods

4.2.1 Animal

Adult female (80-100g) and Juvenile *Penaeus monodon* (30-40 g) were obtained from farm nearby Bangkok, Thailand.

4.2.2 Production of monoclonal antibody

4.2.2.1 Antigen preparation

Adult female *P. monodon* (at resting stage of ovarian development) were obtained from local fisherman in nearby Bangkok, Thailand. The eyestalks were removed from ice-anesthetized animals and dissected the external shell in ice cold 1.8% NaCl. After removal of the external shell, the eyestalk were fixed in Bouin's fixative containing 0.5% glutaraldehyde overnight, removed fixative in running tap water, dehydrated in a graded ethanol series, cleared in xylene and then embedded in paraffin as described in section. Serial sections of 50 μm thick were cut, placed on gelatin-coated slides and baked for 3 days at 50^{o}C prior to deparaffinization. The paraffin sections were deparaffinized in xylene 3 times, xylene:butanol, butanol, a graded ethanol series and water.

The sinus gland from each section was dissected under microscope the wash and homogenized with phosphate buffer saline (PBS) and stored at –70 ^{o}C until use. The position of sinus gland was located by immunohistochemistry of one in ten sections using mouse anti T+ antiserum (against CHH of *Macrobrachium rosebbergii*, Sithigorngul et al., 1999)

4.2.2.2 Immunosuppression and Immunization

Thoracic ganglia of *P. monodon* were dissected and fixed in 0.5% glutaraldehyde overnight. After washing with water thoroughly, the thoracic ganglia were homogenized in PBS. Three swiss mice were injected intraperitoneally with approximately 0.5 ganglian/mouse mixed with complete Freund's adjuvant (1:1). Three days after immunization, all of mice were injected with 100 µl of cyclophophamide (40 mg/kg) in PBS. Three weeks later all three mice were injected four times with approximately 5 sinus gland/ mouse mixed with incomplete Freund's adjuvant (1:1) at two week intervals. A week after the fourth injection, the blood sample was taken from each mouse and the serum was collected. The serum antibody was tested for binding to the sinus gland in eyestalk section by immunohistochemistry. The best mouse with high titer of specific antibody against sinus gland was used as spleen cell donors for hybridoma production.

4.2.2.3 Monoclonal antibody production

4.2.2.3.1 Myeloma cells preparation

A P3X myeloma cell obtained from the Department of Virology, AFRIMS, Bangkok , Thailand was used as the fusion partner. One week prior to fusion, myeloma cells were grown in HT medium with 20% fetal bovine serum (FBS) and incubated in 5% CO_2 at 37^{o}C. When the cells have grown sufficiently, they were subcultured and maintained. One day prior to fusion, the cell were subcultured in fresh HT medium. It is essential that the myeloma cells are in the log phase of growth. This can be ensured by keeping the cell viability of greater than 95%.

4.2.2.3.2 Mouse spleen cell preparation

Three days before fusion, the best mouse was injected with sinus gland. The selected mouse was killed by cervical dislocation. It was soaked rapidly with 70% ethanol and place on it right side. Aseptically remove the spleen, the spleen and abdominal wall were cut. The spleen was removed and washed in twice PBS and HT medium. The small pieces of spleen were mash though a sterile grid using the pluger from syring (10 ml). The spleen cells were used immediately for fusion.

4.2.2.3.3 Hybridoma production

The cell fusion procedure was adapted from the method developed by Kohler and Milstein (1976) with modifications described by Mosman et al.,(1979).

The spleen cells and myeloma cells were mixed and centrifuged at 1,500g for 5 min. The supernatant was removed. The pellet was washed in HT medium and centrifuged. The 1 ml of 40% PEG in HT medium was add to the cell pellet. The mixture was shaked by hand and leave for 1 ml (allow the PEG to coat cells thoroughly), followed by 39 ml HT medium. The tube cell was placed in the incubator for 2 hr after that the tube cell was centrifuged at 1,500g for 5 min and resuspened in HAT medium containing 2% mouse red blood cell. The fusioned cell were placed into 30 micro-culture plate (96 well) with 150 ul per well.

The fifth days, hybridomas should start to become visible by using inverted microscope. After 10-12 day, the hybridomas had grown sufficiently, the culture medium was ready for screening by using immunohistochemistry

The antibody secreting hybridomas from individual wells were future collected, retested the positive clones by limiting dilution and expanded to be frozen.

4.2.2.3.4 Screening for monoclonal antibody by immunohistochemistry

(a) Preparation of paraffin section

The eyestalks were removed from live adult *P. monodon* and dissected the external shell then fixed in Bouin's fixative containing 0.5% glutaraldehyde immediately overnight. The fixed eyestalks were removed fixative, dehydrated in a graded ethanol series, cleared in xylene and then embedded in paraffin by following: washed in tap water several times, soaked in 70%-90% ethanol for 2 hr each, in 95% for 24 hr, in 95%: butanol for 30 min, in butanol for 1 hr, butanol : xylene for 30 min, xylene twice times for 1 hr each, xylene : paraffin for 30 min, paraffin three times for 1 hr each and then embedded in block.

Serial eyestalk sections (5 or 50 μm thickness) were cut from paraffin-embedded block on a microtome, mounted onto gelatin-coated slides and baked for a day at 50^{o}C prior to deparaffinization. The deparaffinization and rehydration of the eyestalk sections were followed by soaking in xylene 3 times, xylene : butanol, butanol, 95% ethanol, 90% ethanol, 70% ethanol and water 3 times for 5 min each, respectively. Sections were blocked in P1+ (10% calf serum in PBS) for 30 min before use.

(b) Immunohistochemistry

Serial sections (5 μm thickness) were prepared and processed for indirect immunoperoxidase staining using various monoclonal antibodies obtained from hybridoma culture medium. Sections were blocked in P1+.

The hybridoma culture medium from each well (see the section 4.2.23.3) were transfer to each section. Sections were incubated for 4 hr at 37^{o}C or overnight at 4^{o}C. After washing in PBS (15 min, 4 times), sections were incubated in secondary antibody (goat anti mouse IgG H and L horseradish peroxidase conjugate ; GAM-HRP) dilute to 1:1000 for 4 hr at 37^{o}C or overnight at 4^{o}C then washed in PBS (15 min, 4 times). Finally slide were developed in a substrate solution containing 0.03% 3,3'-diaminobenzidine 4 HCl (DAB), 0.006% hydrogenperoxide (H_2O_2) in PBS. The immunoreactive sections were counterstained with eosinY, dehydrated in a graded ethanol series, cleared in xylene and mouted in permount. Immunoreaction were visualized as brown coloration against pink background of eosinY.

4.2.2.3.5 Cloning and expansion

After identifying well containing positive clone that produce antibody against sinus gland by immunohistochemistry method. Hybridmas were cloned at least twice by limiting dilution. Cell from the positive well were diluted with HT medium containing 20% FCS and mouse red blood cells were plated out into 96 well plates at the theoretical concentration of 1 cell/well.

Only well containing a single clone after seeding were repeated antibody screening test. Positive hybridomas were propagated in gradually increasing amounts of medium and frozen as soon as possible. The culture medium from each positive clones were collected for characterization of monoclonal antibody.

4.2.2.3.6 Cryopreservation

One of the major reasons for hybridoma production is to insure a continuous supply of specific, reproducible antibody. To safeguard this supply, it is necessary to preserve the hybridomas or myeloma cells at utralow temperatures. First the culture was checked for healthy growth and free from

contamination. The cells were centrifuged at 1500g for 5 min. After the supernatant was removed, the cell pellet was then resuspended in 0.5 ml of 12% dimethysulfoxide and transferred into cryotube. The cryotubes were placed in –70^{o}C overnight and then transferred into liquid nitrogen.

4.2.2.3.7 Thawing

The cryotube cell was removed from the liquid nitrogen and immediately immersed in a 37^{o}C water bath for 2-3 min or until ice is gone. As soon as liquidified, the cryotube was removed and the cells were transferred carefully with pasture pepette in HT medium and centrifuged at 1,500g for 5 min. The pellets were resuspended in HT medium with 20% FCS and incubated at 37^{o}C, 5% C_2O incubator. Growing cell were subculture every 2-3 days.

4.2.3 Characterization of monoclonal antibody

4.2.3.1 immunohistochemical localization of sinus gland peptide

Eyestalks from live adult female *P. monodon* were collected, dissected the external shell then fixed in Bouin's fixative containg 0.5% glutaraldehyde for 24 hr before processing for paraffin sectioning (the 4.2.2.3.4). Serial sections (50 μm thickness) were prepared and process for indirect immunuperoxidase staining using various monoclonal antibodies obtained from culture medium (the section 4.2.2.3.4)

4.2.3.2 Dot-ELISA for RP-HPLC fraction from sinus gland extract

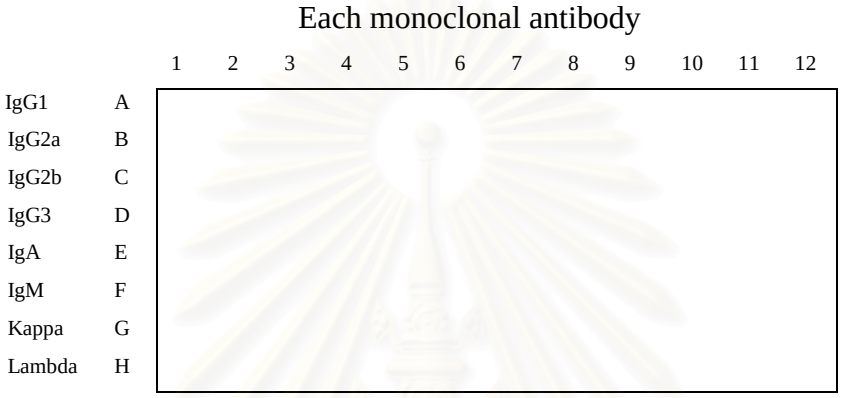
A highly sensitivity dot-ELISA adapted from Sithigorngul et al., (1991) was use to determine the presence of immunoreactive peptides in the fraction as described in the section 4.2.4. Briefly, an aliquot of each fraction (approximately equivalent to 20-40 eyestalks/spot) was mixed with 1 mg/ml of bovine serum albumin (BSA) dissolved in PBS at the proportion that yield 1ug BSA/spot. The mixture was

then dried in the vacuum concentrator (Savant). Distilled water (1ul/spot) was added to dissolve the mixture and then transferred as a spot on a nitrocellulose membrane. After the membrane was air dried and baked at 60^{o}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 distilled water for 15 min. The membrane was washed thoroughly with distilled water and blocked 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 diluted of various monoclonal antibodies. After washing with 0.5%blotto (15 min, 4 times).The membrane was then incubated for 8 hr in GAM-HRP diluted 1:1000 with 5% blotto . The membrane was washed with 0.5% blotto (15 min, 4times) the membrane was color developed in a substrate solution containing 0.03% DAB, 0.06% H_2O_2 and 0.05% $CoCl_2$ in PBS for 3-5 min and wash thoroughly in distilled water. The immunoreactive spots were observed as gray-black coloration.

4.2.3.3 Class and subclass determination

Class and subclass of mouse immunoglobulins produced by hybridomas were determined by sandwich ELISA using Mouse Mono Ab kit (HRP; Zymed). The wells of 96 well plate were coated with 50 µl of goat anti- mouse IgG+IgA+IgM at 1:100 diluton and incubated overnight at 4^{o}C. The plate was washed with 0.5% blotto (10 min, 4 times) and blocked in 5 % blotto for 30 min. Each of MAb (in culture medium) was added to wells in each column and incubated for 8 hr at room temperature. After washing, the 50 µl of rabbit anti mouse IgG1, IgG2a, IgG2b, IgA, IgM, lambda and kappa light chain at 1:20 dilution was added to well in row A-H respectively (see below). Following the incubation period, the plate was wash with 0.5% blotto (10min, 4 times) and then incubated with goat anti rabbit IgG H+L horseradish peroxidase conjugate (GAR-HRP) at 1:1000 dilution for 8 hr at room

temperature. The plate was washed with 0.5% blotto (10 min, 3 times and then PBS. The plate was developed color in a 80 ul of substrate solution containig 1 mg/ml o-phenelene diamine dihydrochoride (OPD), 0.06% H_2O_2 in 0.1 M citrate buffer, pH4.5. The reaction was stopped by adding 80 µl of 1N sulfuric acid (H_2SO_4). The optical density of each well was determined at 490 nm using microplate reader.



4.2.4 Isolation of immunoreactive peptide

4.2.4.1 Preparation of sinus gland extract

(a) sinus gland collection

Eyestalk was excised from live juvenil *P.monodon* (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 ^{o}C until used.

(b) Peptide extraction

Approximately 2500 sinus glands were homogenized in 50 ml extraction solution on ice. After incubation at 4^{o}C for 2 hr, the extraction was centrifuged at 10,000g for 30 min. The supernatant and the pellet were separately re-extracted with 25 ml extraction solution. After combining the supernatant, methanol

and acetic acid was eliminated using a speed vacuum concentrator then adjusting the solution to final concentration of 0.1%trifluoroacetic acid (TFA) for a final volume at 3 ml. The extract was then passed through C18 SepPak cartridge and eluted with 50% acetonitrile in 0.1% TFA. The elute was concentrated by seed vacuum concentrator. A final concentration of the extract was diluted with 10% acetonitrile in 0.1% TFA

4.2.4.2 Chromatography

The sinus gland extract was centrifuged (10,000g, 15 min) to remove undissolved material. Separations were performed on a Gilson HPLC system and monitored at 215 nm with Gilson 119 UV detector. The fractions were collected at 1 ml/min and aliquoted for identification of peptide recognized by different monoclonal antibodies separately using dot-ELISA. The steps of separation are as follows:

Step1 The sample was applied to C18 column (4.6x250mm, Vydac) and eluted using a linear gradient, 8-64% solvent B with flow rate 1 ml/min ; solvent A is 0.1% TFA and solvent B is 80% acetonitrile in 0.1% TFA.

Step2 The immunoreactive fraction from step1 were applied to C 8 column (4.6x250 mm, Rainin Instrument) and eluted using a linear gradient 16-48% solvent B (flow rate 1 ml/min) ; solvent A is 0.1% heptafluorobutyric acid (HFBA) and solvent B is 80% acetonitrile in 0.1% HFBA.

Step3 The immunoreactive fraction from step were applied to cyano column (4.6x250 mm, Rainin Instrument) and eluted as in step1.

4.2.4.3 Peptide detection

Dot-ELISA was used throughout to detect the immunoreactive peptide in the fractions during the separation process. Briefly, an aliquot of each sample (approximately content equivalent to 50-200 sinus gland /spot) was mixed with 1

mg/ml of BSA dissolve in PBS at proportion that yielded 1 μg BSA/spot. The mixture was then dried in the vacuum concentrator. Distilled water was added to dissolve the mixture and then 1 μl of the mixture was transferred to a spot on a nitrocellulose membrane. The processed for dot-ELISA as described in section 4.2.3.2. The immunoreactive fractions were pooled subjected to next step of purifications until separated peaks were obtained.

4.2.2.4 MALDI-TOF mass spectrometry

Matrix-assited laser desorption ionization time of flight mass spectrometry was performed on a BIFLEX MALDI-TOF from Bruker-Franzen Analytik GMBH. Approximately 10% of each purified fraction was eused for molecular mass determination. The purified fraction was dried in the vacuum concentrator and resuspened in a 3 ul solution of 33% acetonitrile in 0.1% TFA. One μl of this sample was mixed with 1 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile. One μl of the mixture was applied to the probe and dried by air. The instrument was calibrated using external standards: human angiotensin II (MW. 1047 daltons) and insulin bovine (MW. 5734 daltons).

4.2.4.5 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 and applied Biosystem Procise Sequencers.

4.3 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 yielding 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. 4.1, Table 4.1). 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 different from sub-populations recognized by the other three antibodies (Fig. 4.1). 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 fractions except for two fractions (46-47) were bound by SG 24 and SG 26 (Fig. 4.3). All fractions were also recognized by anti-T+ antiserum, the antiserum made against C-terminal peptide of CHH from *Macrobrachium rosenbergii* (Sithigorngul et al., 1999). The other antibody (SG 782) bound to fractions 26-28 (Fig. 4.3). The peptides from these fractions were further purified by one or two more steps of RP-HPLC (Fig. 4.4-4.5) and using monoclonal antibodies specific to those fractions to monitor the peptides during purification until the relatively pure peptides were obtained (Fig. 4.4-4.5). 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 (SG 26-4) of the eight peptides recognized by SG 26 antibody (from fractions 48-51) were subjected to sequence analyses (Fig. 4.6). The N-terminal sequence of the three peptides range from 37-50 residues revealed similarity to the N-terminal sequence of CHHs, Pem-Sgp I, Pem-Sgp III and PemSgp IV identified by Davey et al., 2000. (Table 4.1 and Fig. 4.6)

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. 4.6). Since the yield of the peptide was low, approximately 5 pmole it was impossible to identify the whole sequence and the amino acids at several positions were still uncertain.

Table 4.1. 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	CHH 8341.18 SgpIII (8349.9)
SG 26	IgG1	~35	+++	44-45 43-53	1 (6505.57)* 2 (3903.24)* CHHs 3 (8223.22)* 4 (8296.35) SgpIV (8294.99) 5 (8426.32)* 6 (8212.48)* 7 (8290.47)* 8 (8208.86)*
SG 293	IgM	~30	+++	40-43	CHH (8522.8) SgpI (8511.05)
SG 782	IgM	2-3	+	26-28	Unknown (9127.56)

* amino acid sequence was not determined

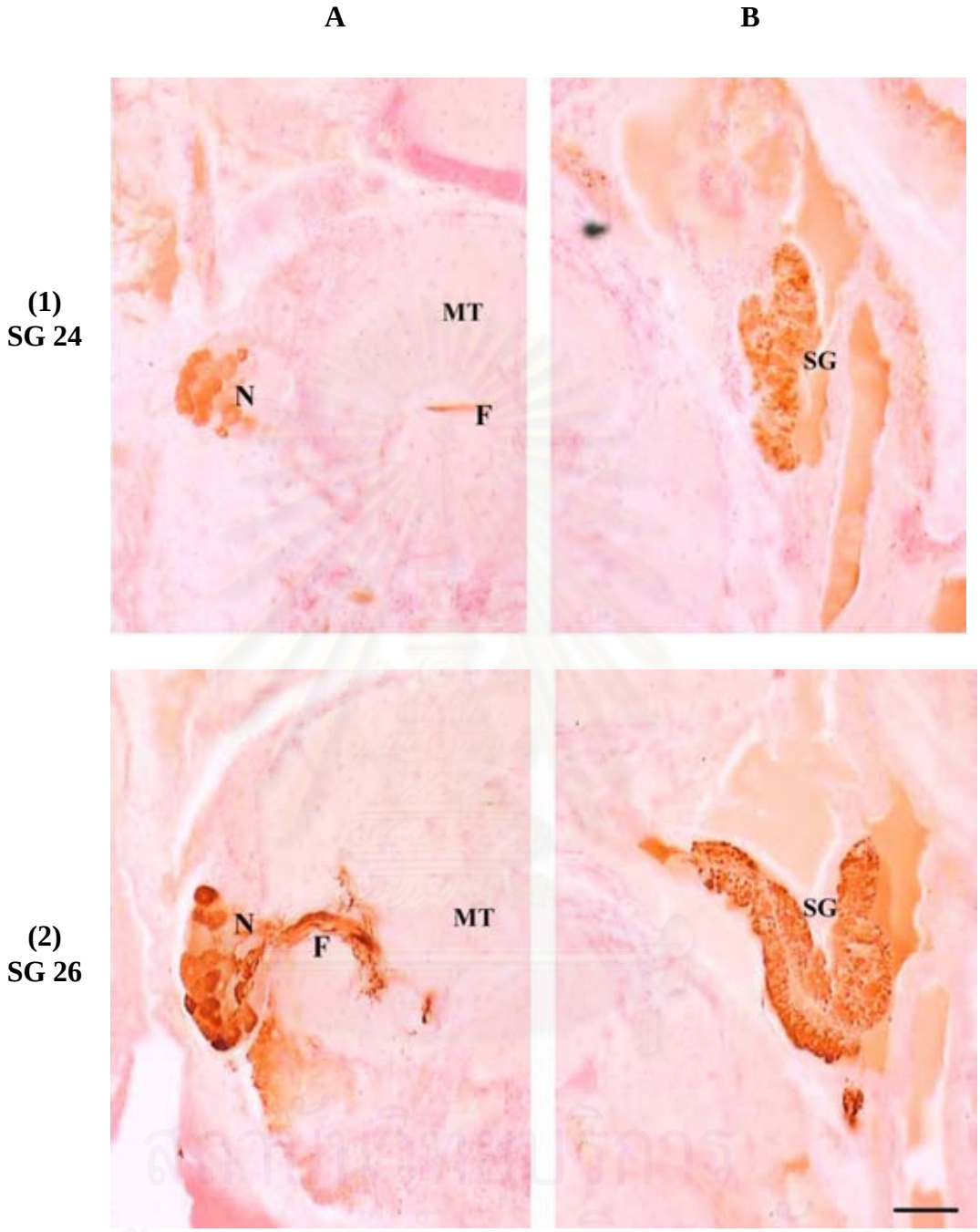
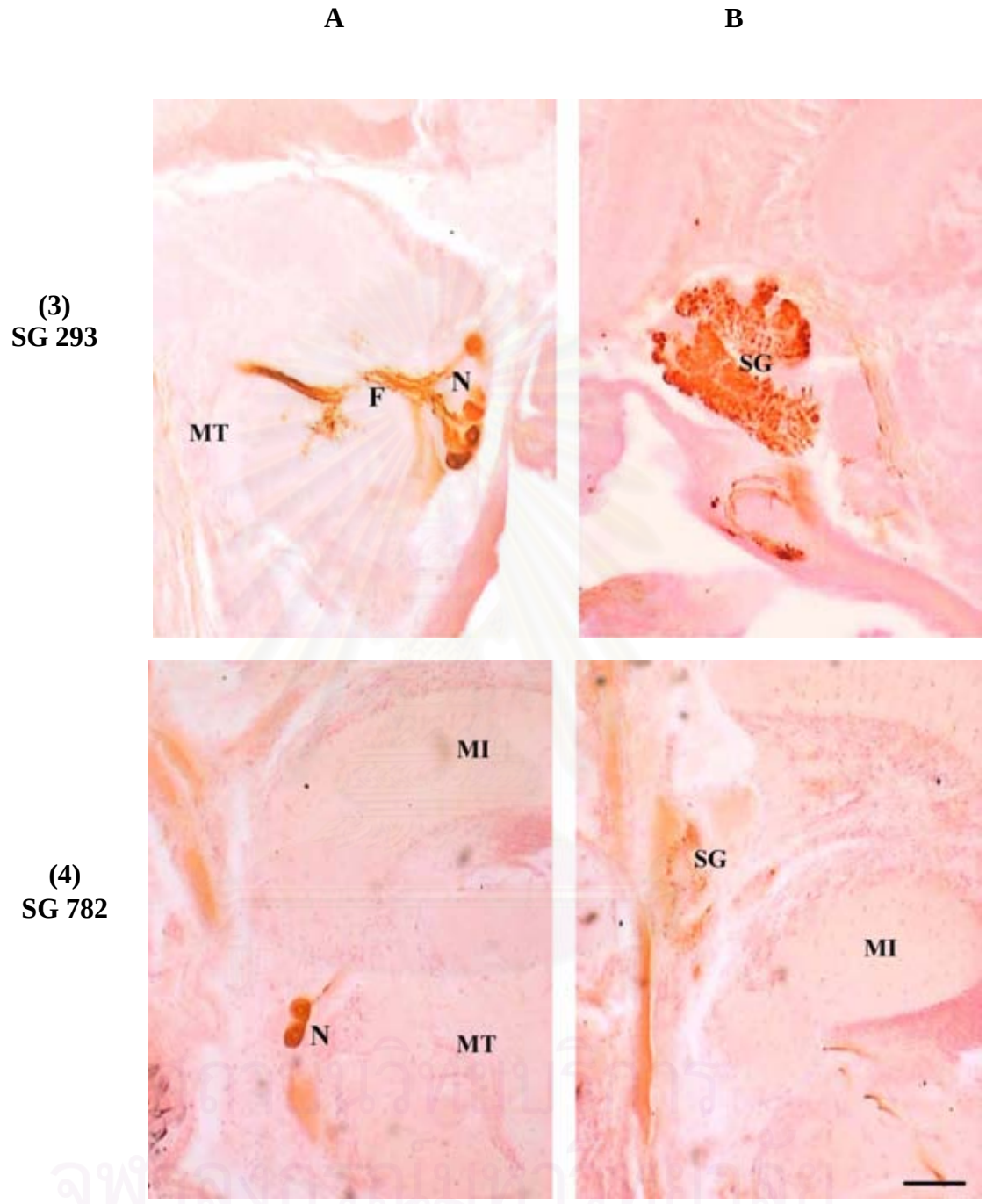


Figure 4.1 Immunohistochemiscal localization of the peptides recognized by monoclonal antibodies; (1) SG24 (2) SG26 (3) SG293 (4) SG782. All antibodies recognized neuronal cell bodies (N) in the MTXO (A) and sinus gland (B). ME = medulla externa, MI = medulla interna, MT = medulla terminalis, SG = sinus gland and F = nerve fiber



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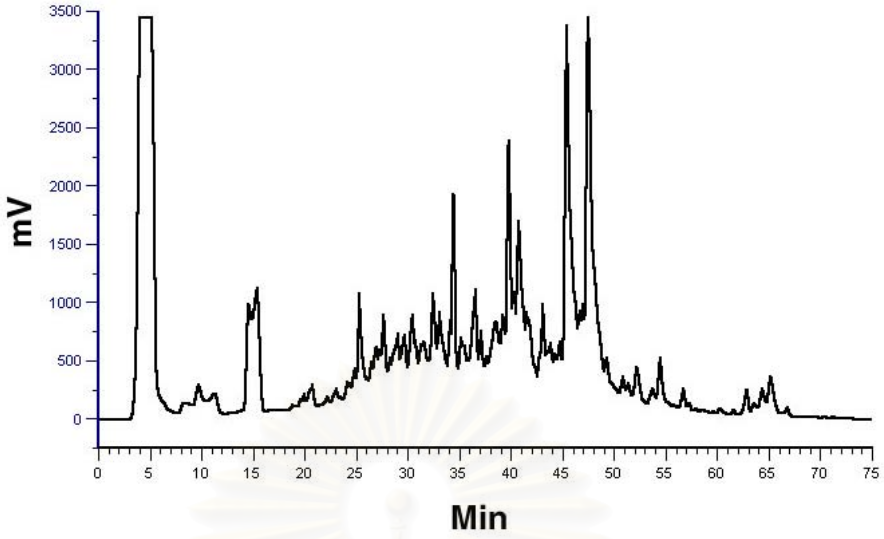


Figure 4.2 The chromatogram of the 1250 sinus gland extract at the first step of RP-HPLC separation

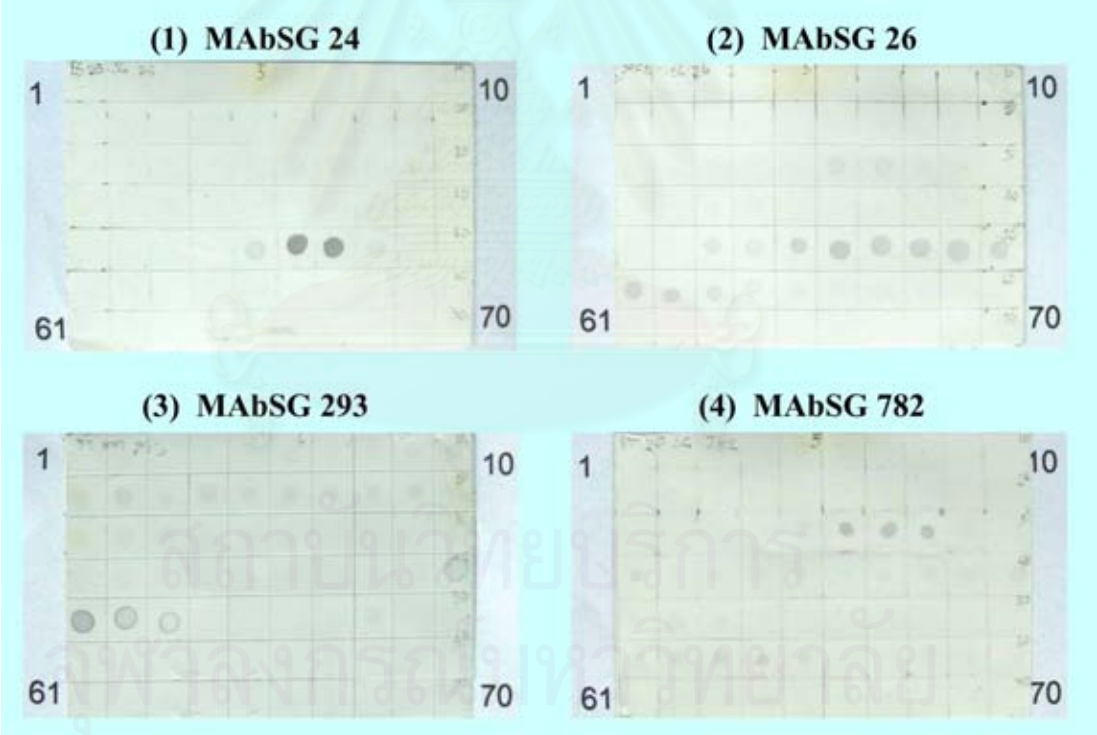


Figure 4.3 Dot-ELISA of the first step fractions treated with monoclonal antibodies; (1) SG24 (2) SG26 (3) SG293 (4) SG782. The fractions were collected at one minute intervals. The indicated number are the fraction numbers and only showed at the first and the last fractions on the top and the bottom rows.

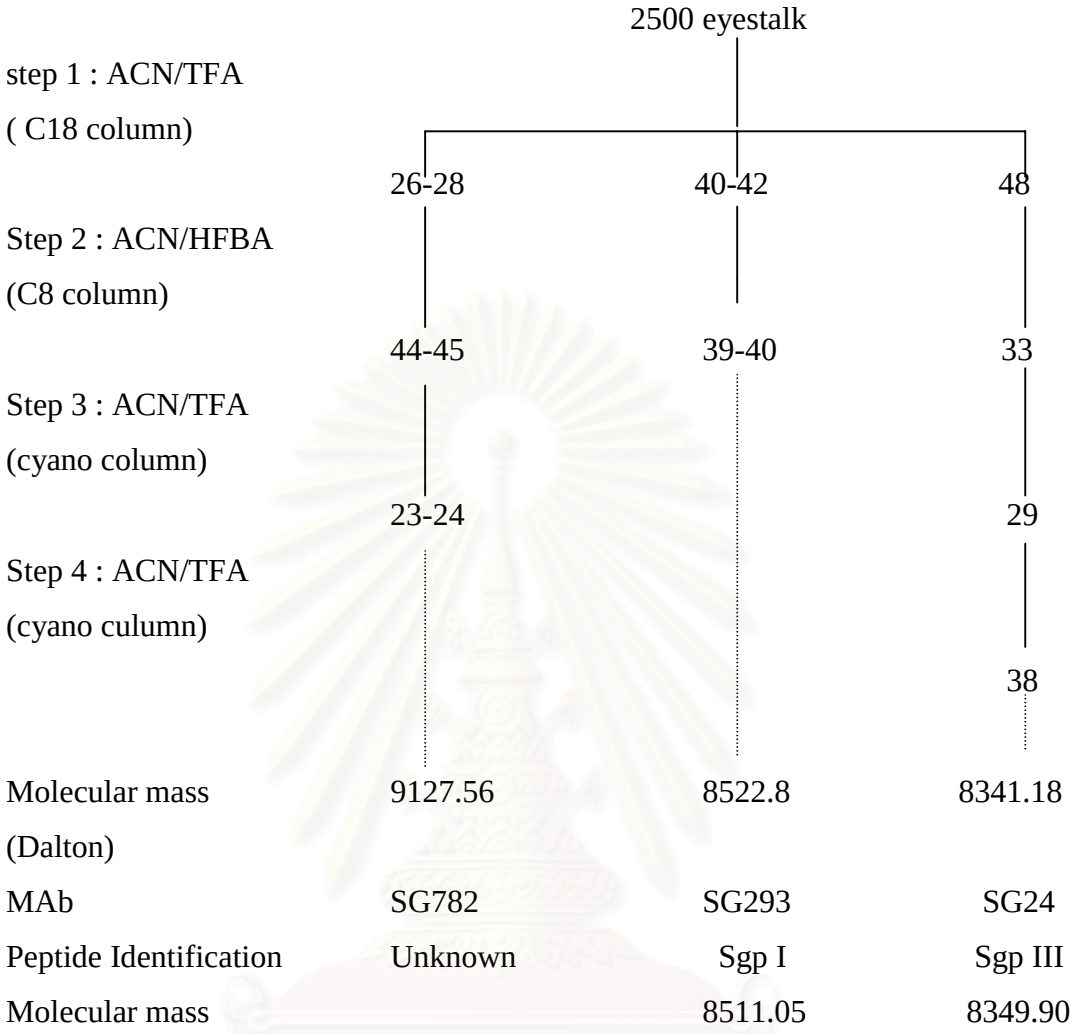


Figure 4.4 Purification steps of the peptide recognized by monoclonal antibodies; SG782; SG293; SG24

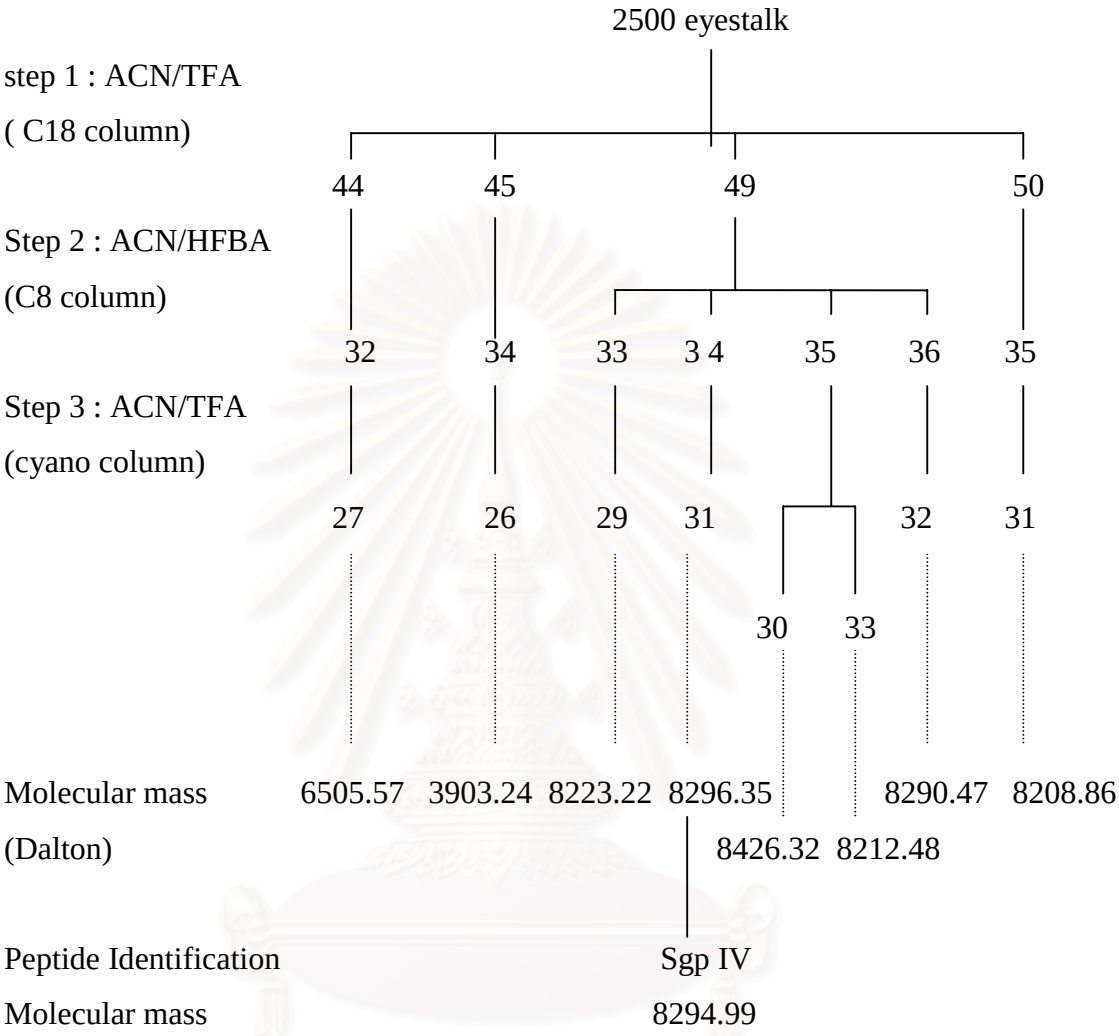


Figure 4.5 Purification steps of the peptide recognized by monoclonal antibody SG 26

1

```
SG 24    ANFDPSCAGV YNRELLGRLS RLCDDCYNVF REPKVATXCR NXCFYNPVFV QCLXY
Sgp III ANFDPSCAGV YNRELLGRLS RLCDDCYNVF REPKVATECR NNCFYNPVFV QCLEYLIPAD
         LHEEYQAHVQ TVGK
```

2

```
SG 26-4 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
```

Figure 4.6 Amino acid sequences of the 4 peptides (1-4) isolated by each monoclonal antibodies and compared with the known peptides. The letters in the parenthesis are minor amino acid presence in that cycle. X = unknown. Underlined residues are the different amino acid compositions.

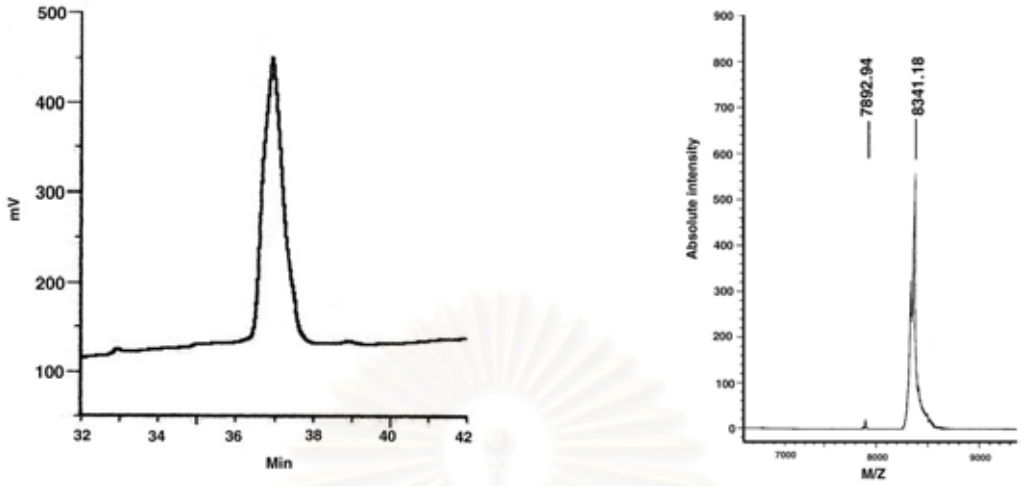


Figure 4.7 Chromatogram and MALDI-TOF MS analysis of purified peptide recognized by monoclonal antibody SG24

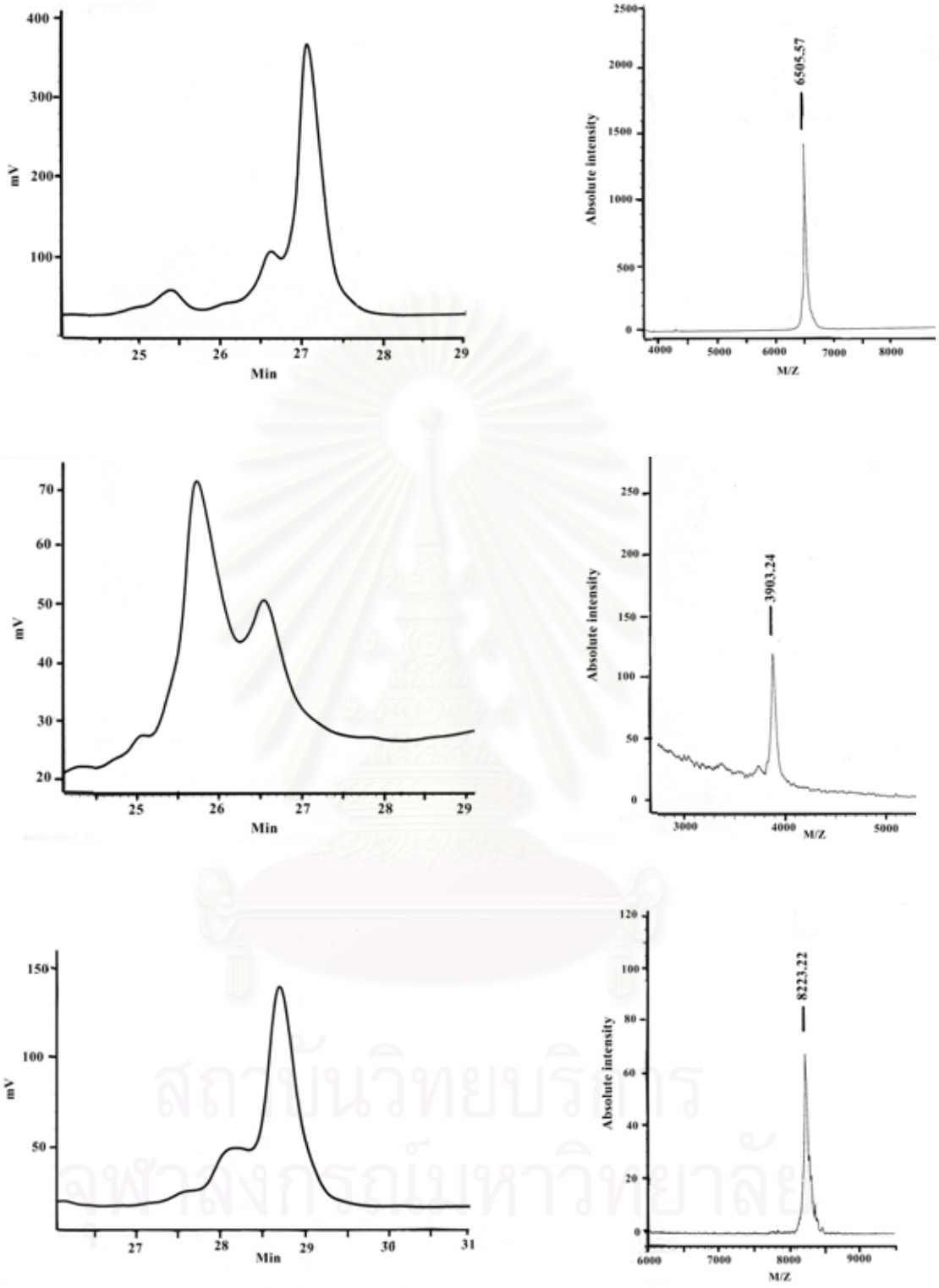


Figure 4.8 Chromatogram and MALDI-TOF MS analysis of purified peptide recognized by monoclonal antibody SG26

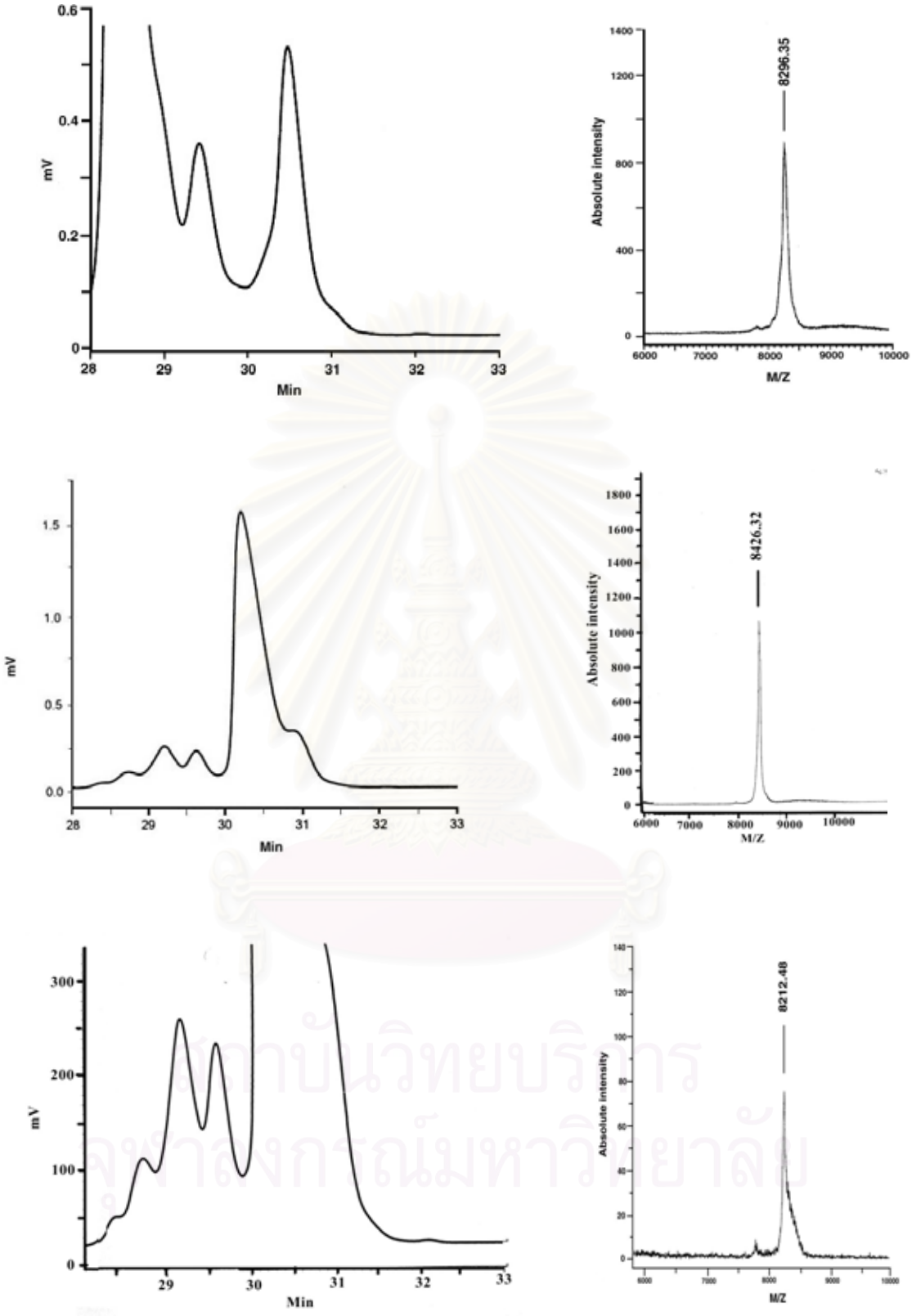
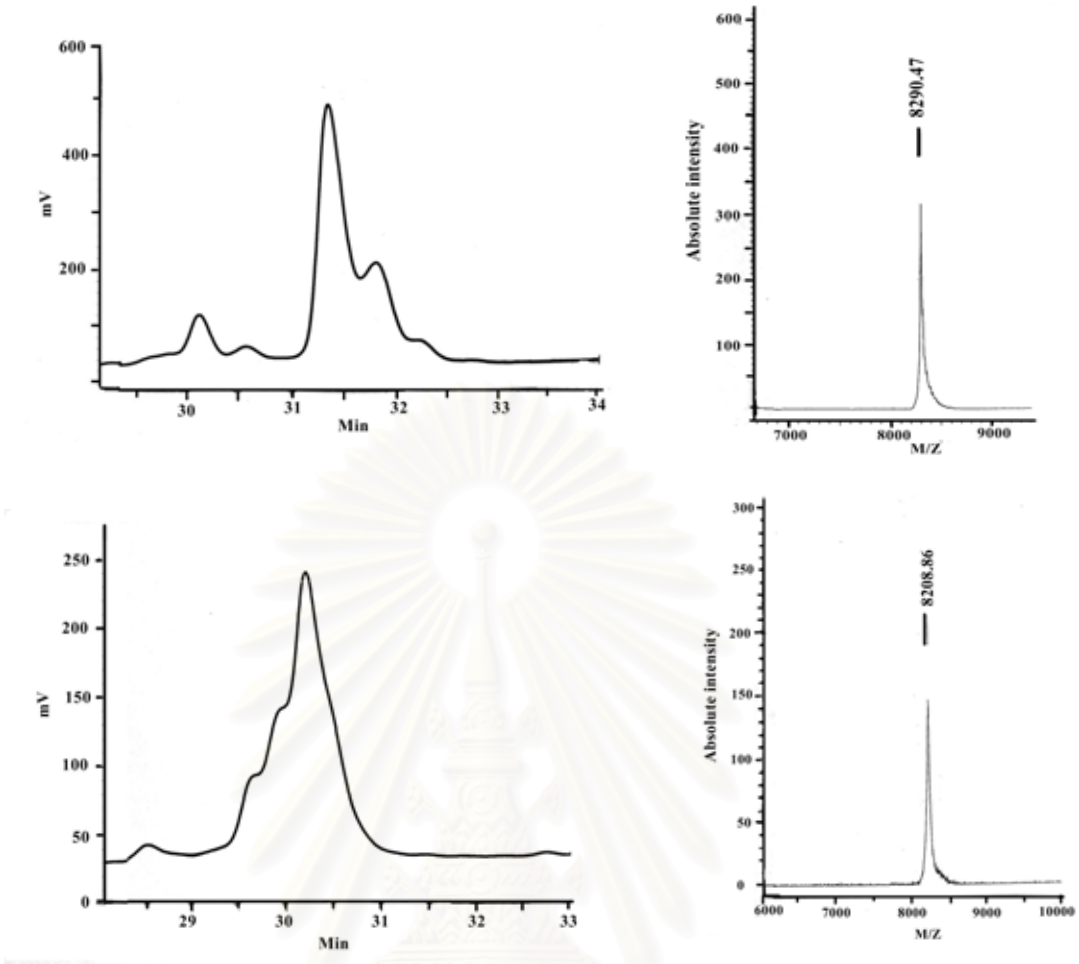


Figure 4.8 Continued



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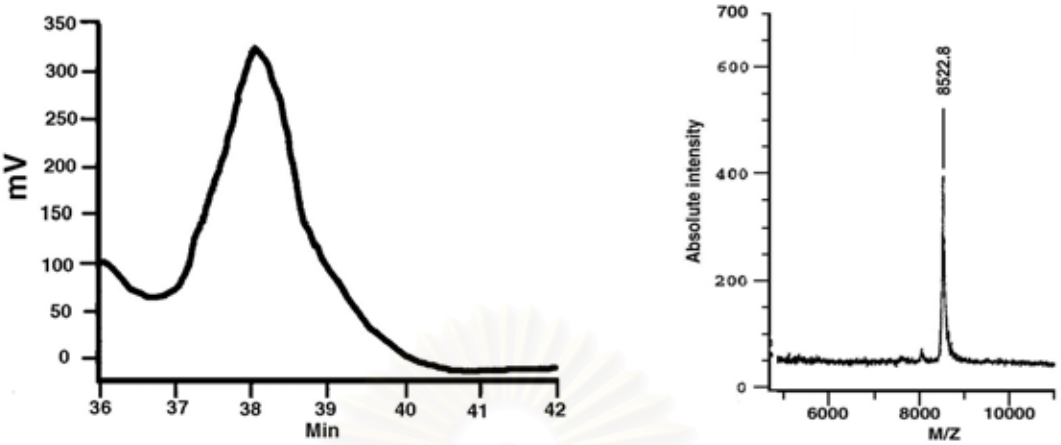


Figure 4.9 Chromatogram and MALDI-TOF MS analysis of purified peptide recognized by monoclonal antibody SG293

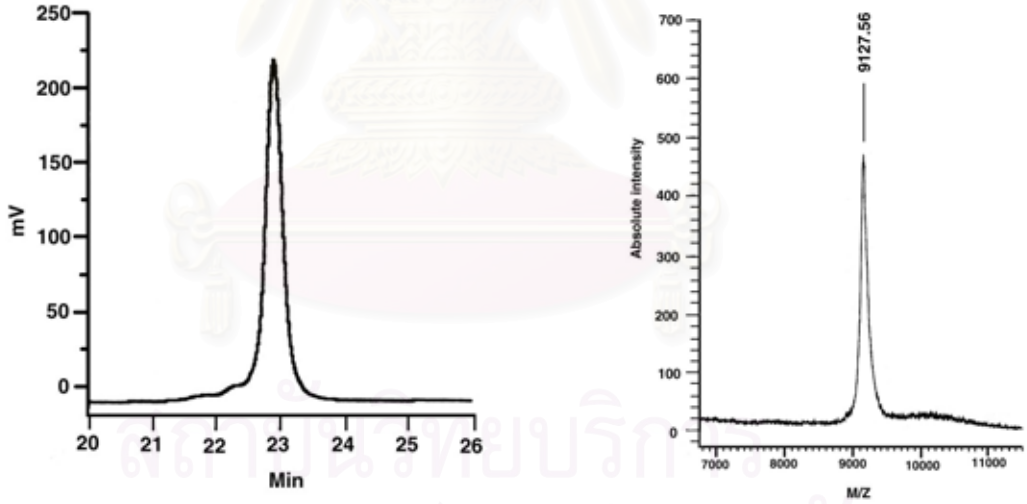


Figure 4.10 Chromatogram and MALDI-TOF MS analysis of purified peptide recognized by monoclonal antibody SG782

4.4 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 (Krungkasem et al., 2002) CMG peptide (Udomkit et al., 2000) FMRFamide peptide family (Sithigorngul et al., 2002a) and NPY/PP family (Sithigorngul et al., 2002b). 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 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, indicated that most of the CHH containing neurons could selectively expressed some of the CHH isoforms.

Interestingly, an antibody specific to an unknown peptide (SG 782) is a IgM, 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 does 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 (Sithigorngul et al., 2003). 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 may allow for molecular cloning of the gene specifying this peptide. Molecular cloning of this peptide using cDNA library of *P. monodon* eyestalk is in progress.

CHAPTER V

Monoclonal antibody against other sinus gland neuropeptides with partially characterized specificity

5.1 Introduction

Four monoclonal antibodies obtained in the previous study can be used to purify peptides belong to CHH family. In this chapter we can isolate other monoclonal antibodies against various neuropeptides in the eyestalk of *P. monodon*. However, the specificity of the monoclonal antibodies are still not well characterized.

5.2 Materials and methods

The same as in chapter IV

5.3 Result

Sinus gland of *P. monodon* from paraffin eyestalk section was used to immunized into mice. The best performing mouse with high titer of antibody against sinus gland was used as spleen donor for hybridoma production. From five fusions, other than SG24, 26, 293 and 782 monoclonal antibodies obtained in previous chapter, eight established hybridoma clones producing specific monoclonal antibodies were isolated. Characterization of monoclonal antibodies by immunohistochemistry in eyestalk and dot-ELISA of sinus gland peptides fraction separated by one step of RP-HPLC revealed that all antibodies designated SG706, 813, 843,1012, 1134, 1251 and 1459 recognized sinus gland and different number of neurons in medulla terminalis X-organ complex (MTXO) in the eyestalk of *P. monodon* (Fig. 5.1 and table 5.1). With dot-ELISA (Fig. 5.2), most monoclonal antibody showed low affinity with sinus gland extract fraction except SG706, SG813 and SG 843 monoclonal antibodies that

have sufficient affinity to monitor the isolated sinus gland peptides during purification (Table 5.1 and Fig. 5.3-5.4).

Five purified peptides from fractions 50-58 recognized by SG 813 monoclonal antibody with molecular mass range from 3500-3700 Da were obtained. One of these (molecular mass 3648.00, SG 813-4) were subjected to sequence analysis. The N-terminal sequence of this first 30 residues revealed similarity to part of the PDH-precursor related peptide in *P. japonicus and P. vannamei* (Desmoucelles-Carete et al., 1996; Ohira et al., 2002) (Fig. 5.7). Two purified peptides from fractions 38-39 recognized by SG 843 monoclonal antibody have the molecular mass of 8525.94 and 8527.31 Da. We did not perform sequencing due to the molecular mass is close to CHH and the peptides were elute in between CHH fractions which previously identified. The peptide from fractions 42-43 recognized by SG706 monoclonal antibody, could not be isolated due to the low yield and could not be purified further.

Table 5.1 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 706	IgM	~7	+	42-43	
SG 813	IgG1	~40	+++	50-58	1 (3549.97)* 2 (3553.75)* 3 (3560.40)* 4 (3648.00) PDH-PPRP 5 (3725.09)*
SG 843	IgM	5-10(3ES) ♦	+++	37-42	(1) 8525.94* (2) 8527.31*
SG 1012	IgM	~23	+	63-68	
SG 1134	IgM	8(3ES) ♦	++	26-28	
SG 1251	IgM	7-9(3ES)♦	+*	26-27, 40-42	
SG 1310	IgM	3-7(3ES)♦	+	26-28, 42-45 51-59	
SG 1459	IgM	8(1ES)♦	+	26-28, 45-49	

♦Detection of 10 eyestalk found immunostaining neurons in some eyestalk ;

ES = eyestalk

* Amino acid sequence was not determined

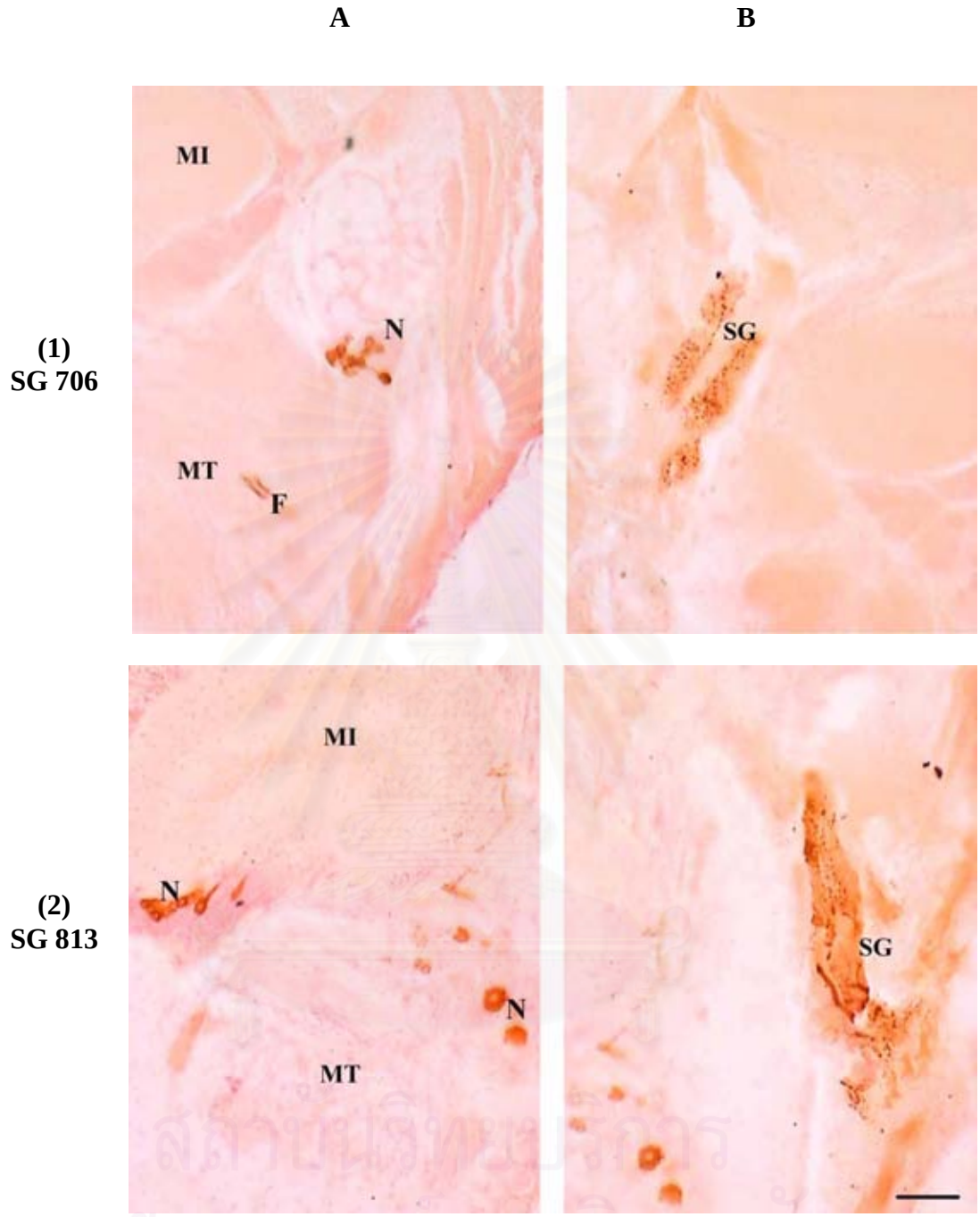


Figure 5.1 Immunohistochemiscal localization of the peptides recognized by monoclonal antibodies; (1) SG706 (2) SG813 (3) SG843 (4) SG1012 (4) SG1134 (6) SG1215 (7) SG1310 (8) SG1459. All antibodies recognized neuronal cell bodies (N) in the MTXO (A) and sinus gland (B). ME = medulla externa, MI = medulla interna, MT = medulla terminalis , SG = sinus gland and F= nerve fiber

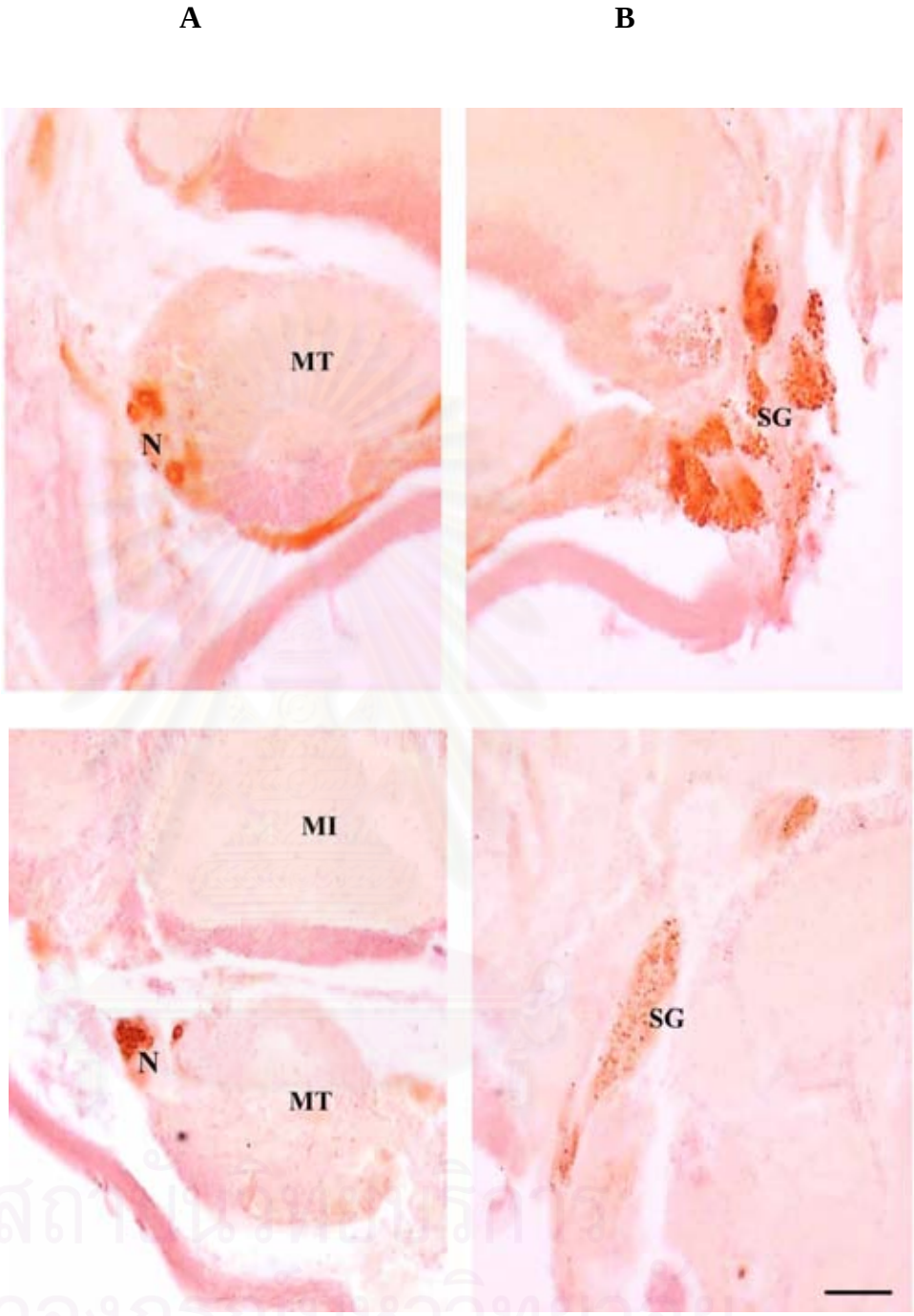
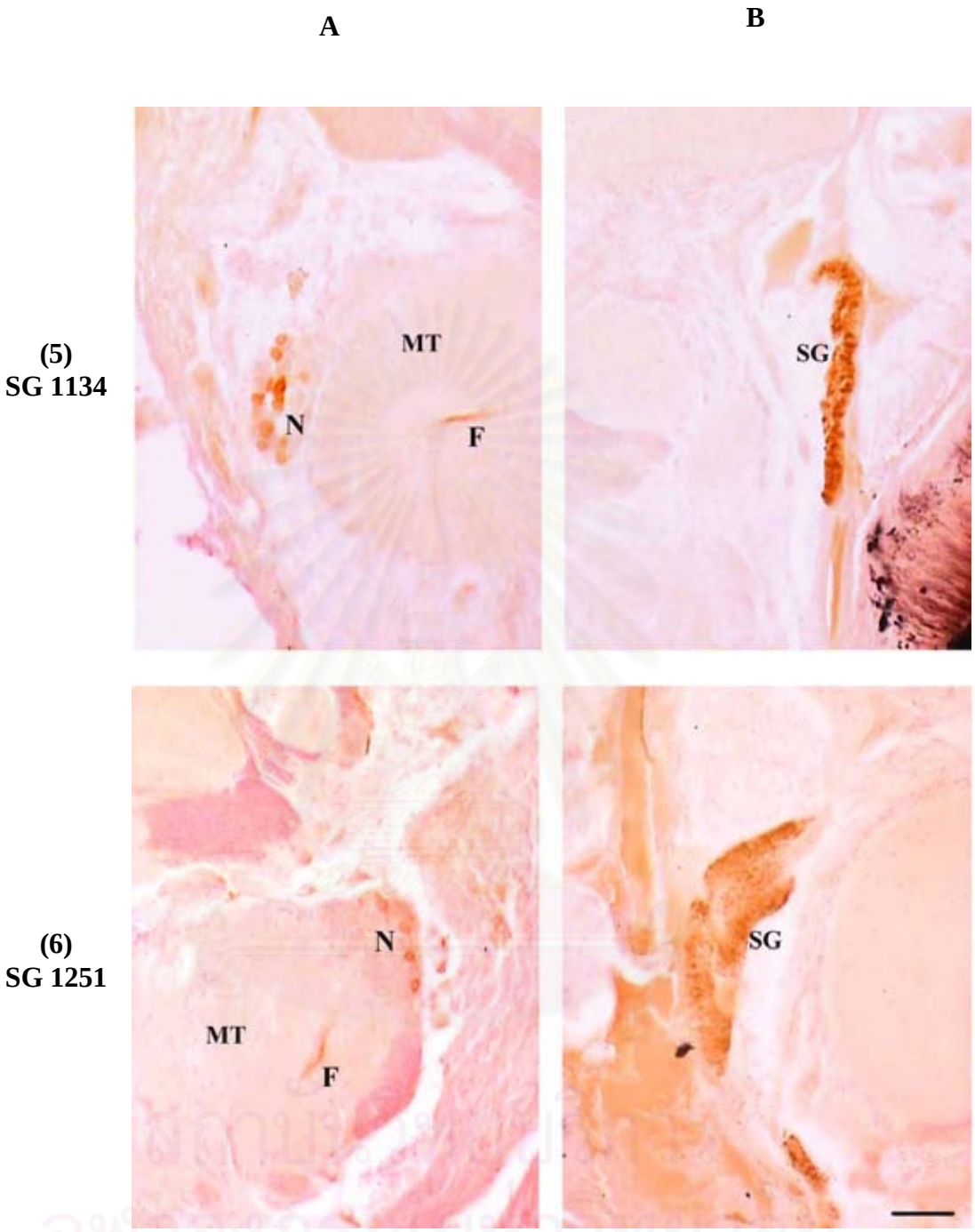


Figure 5.1 continued



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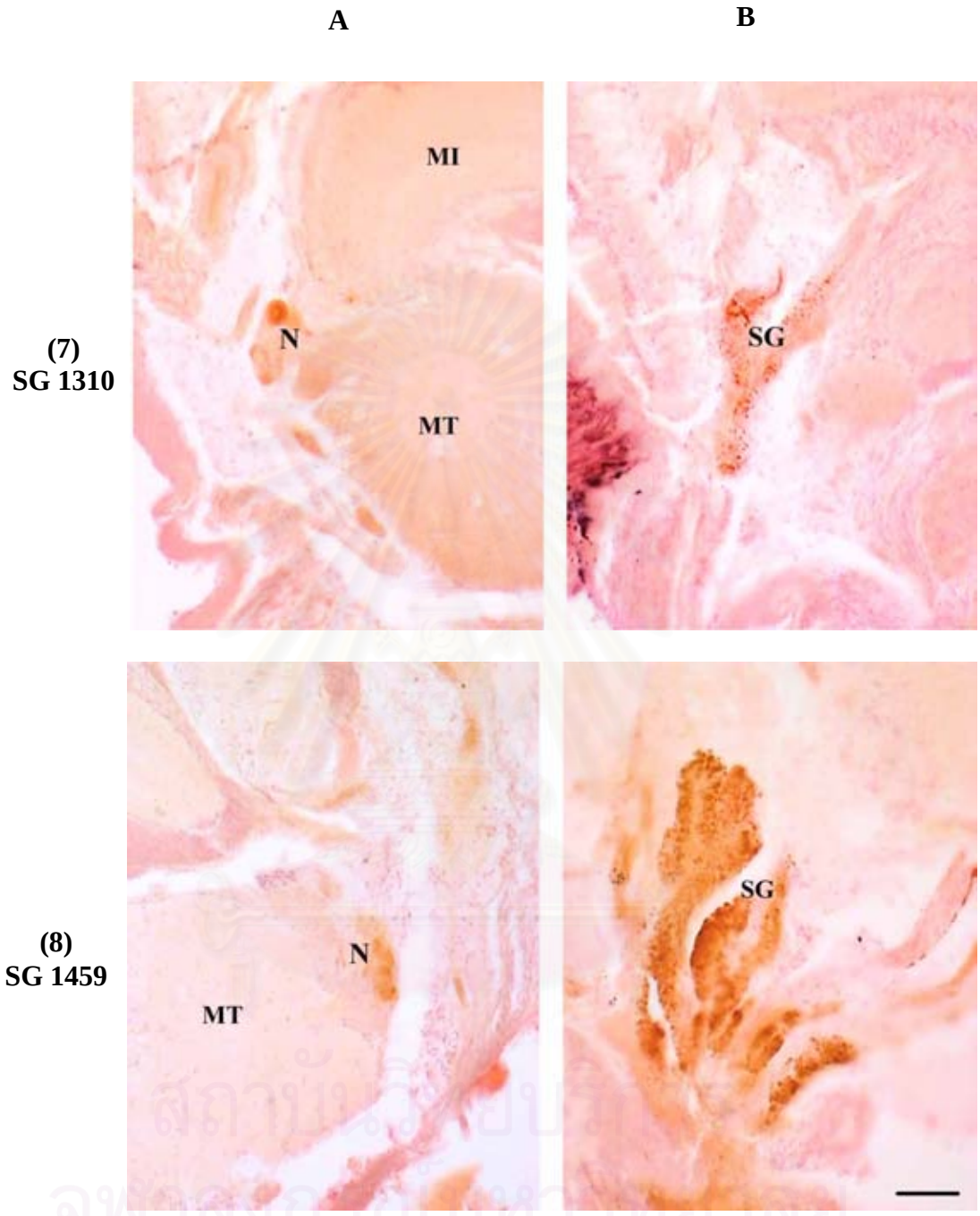

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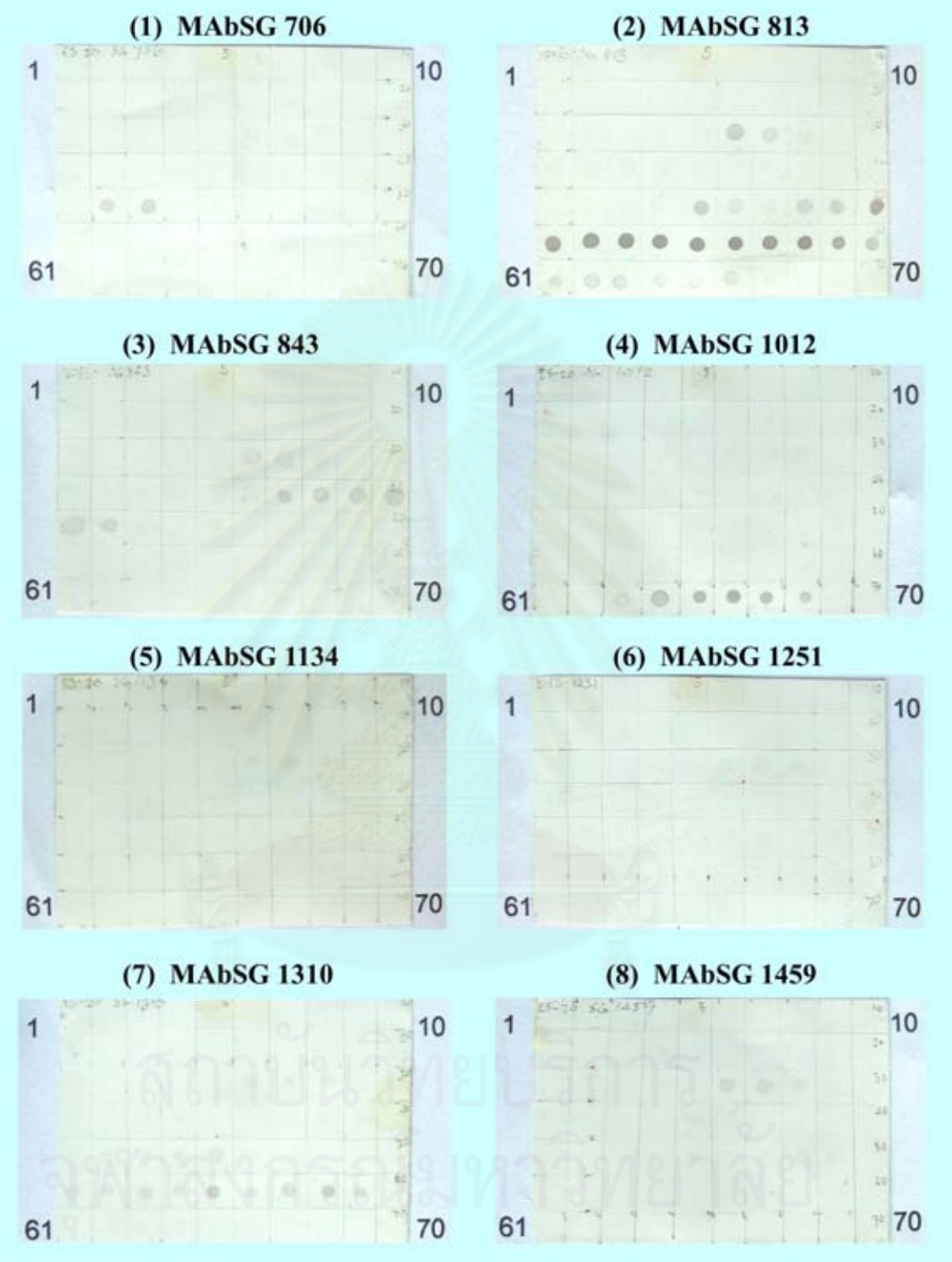


Figure 5.2 Dot-ELISA of the first step fractions treated with monoclonal antibodies; (1) SG706 (2) SG813 (3) SG843 (4) SG1012 (5) SG1134 (6) SG1215 (7) SG1310 (8) SG1459. The fractions were collected at one minute intervals. The indicated number are the fraction numbers and only showed at the first and the last fractions on the top and the bottom rows.

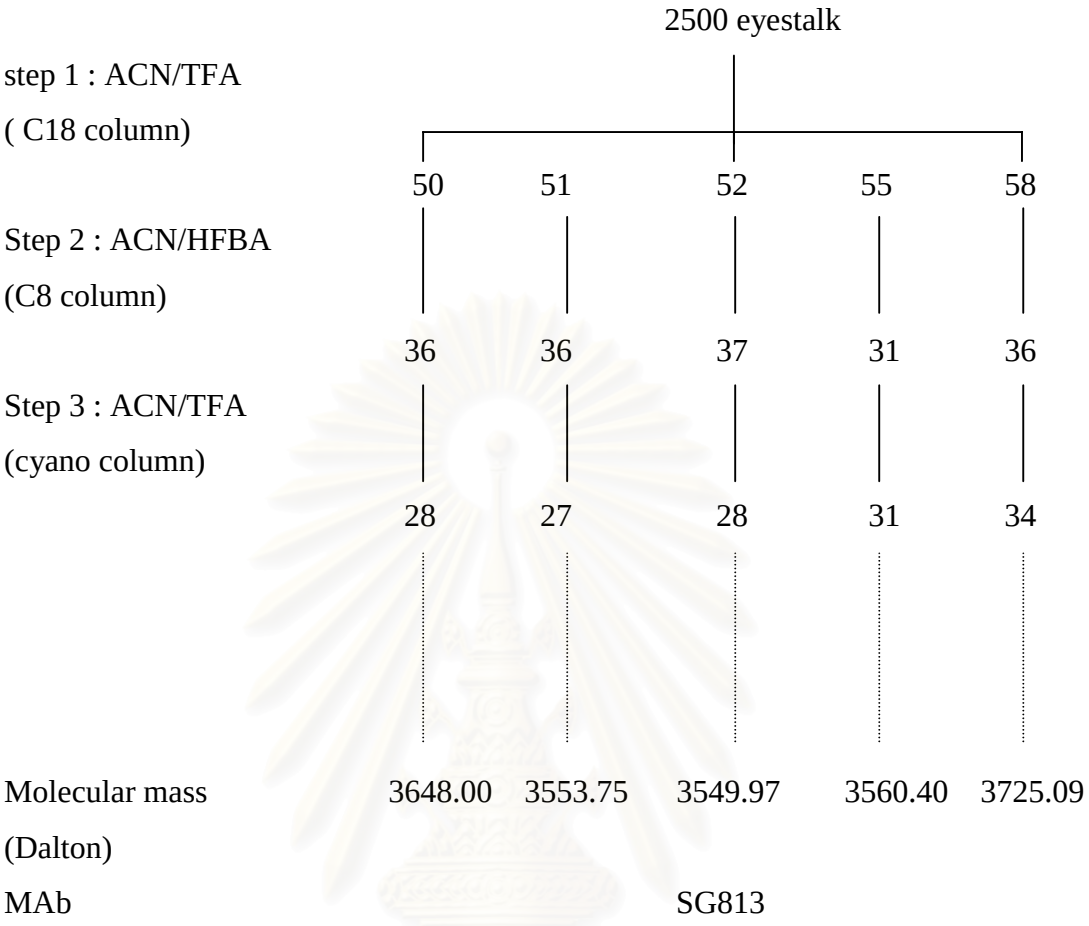


Figure 5.3 Purification steps of the peptide recognized by monoclonal antibody SG813

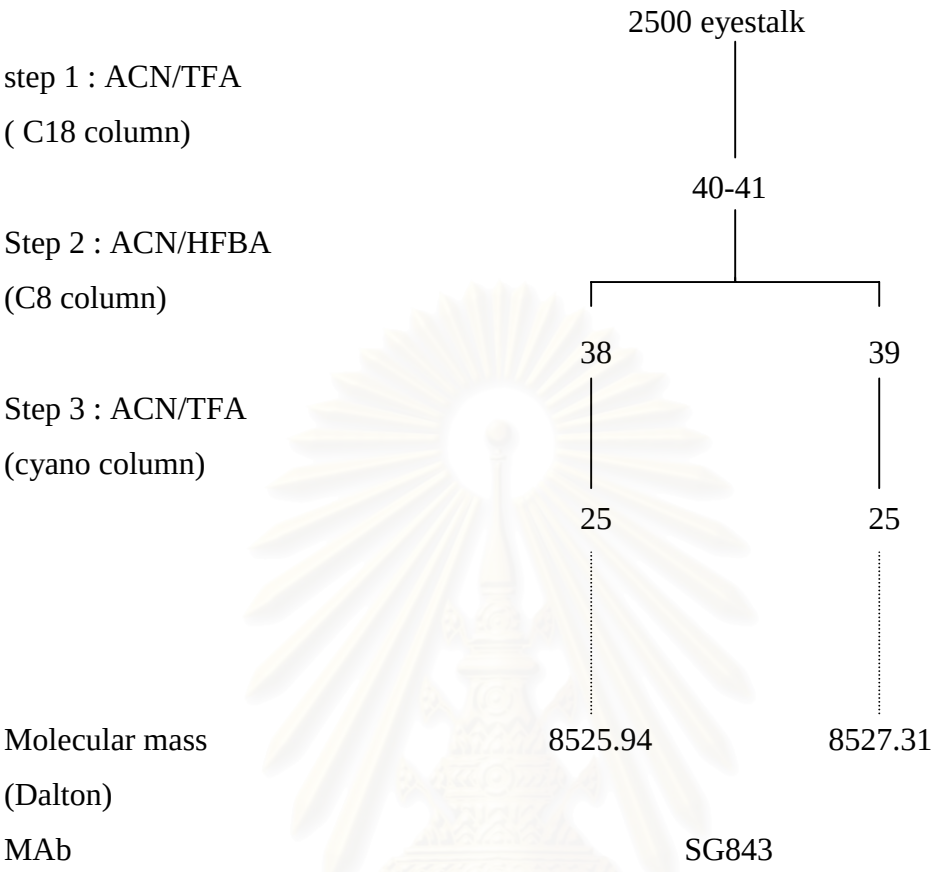


Figure 5.4 Purification steps of the peptide recognized by monoclonal antibody SG843

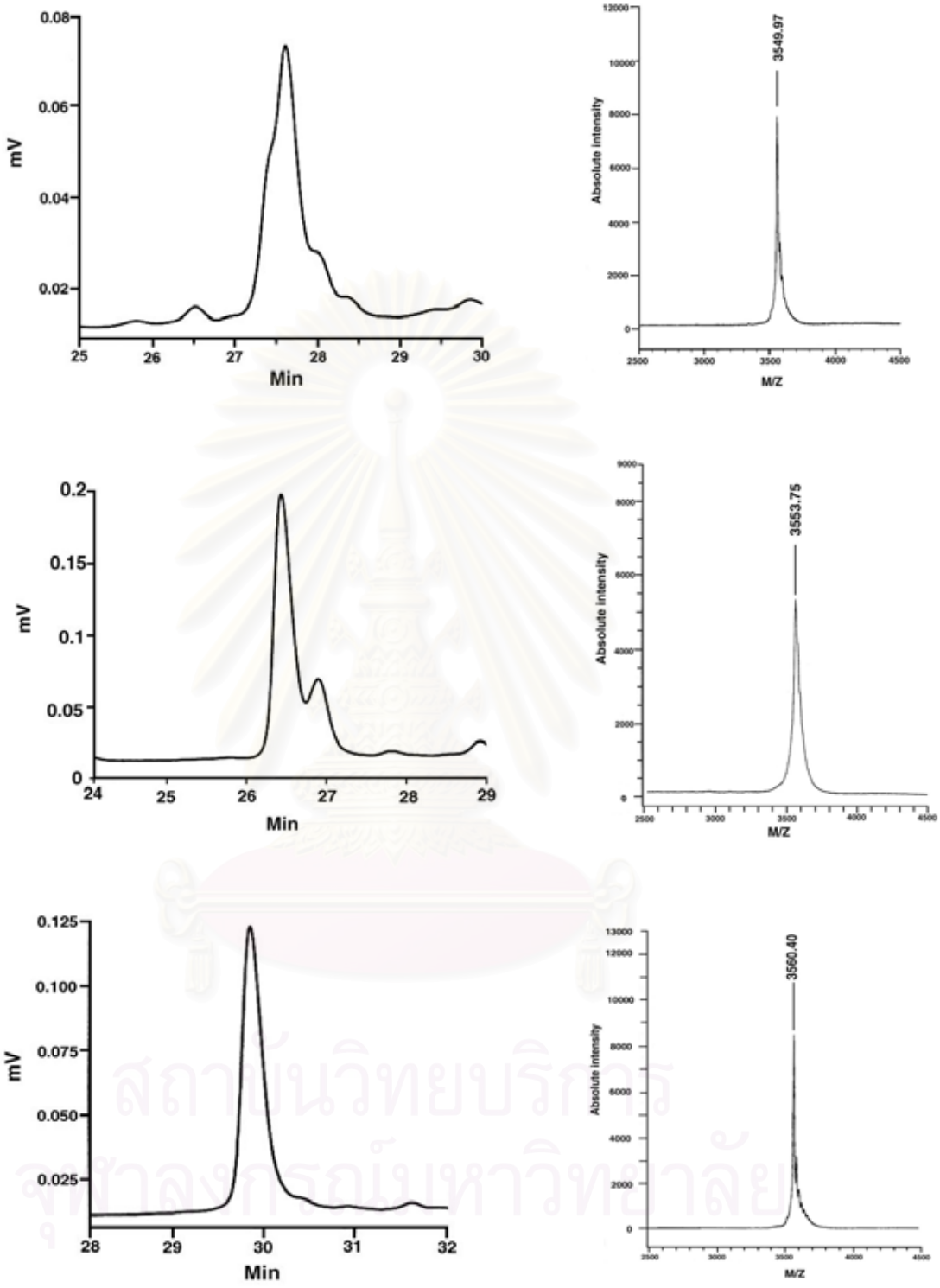
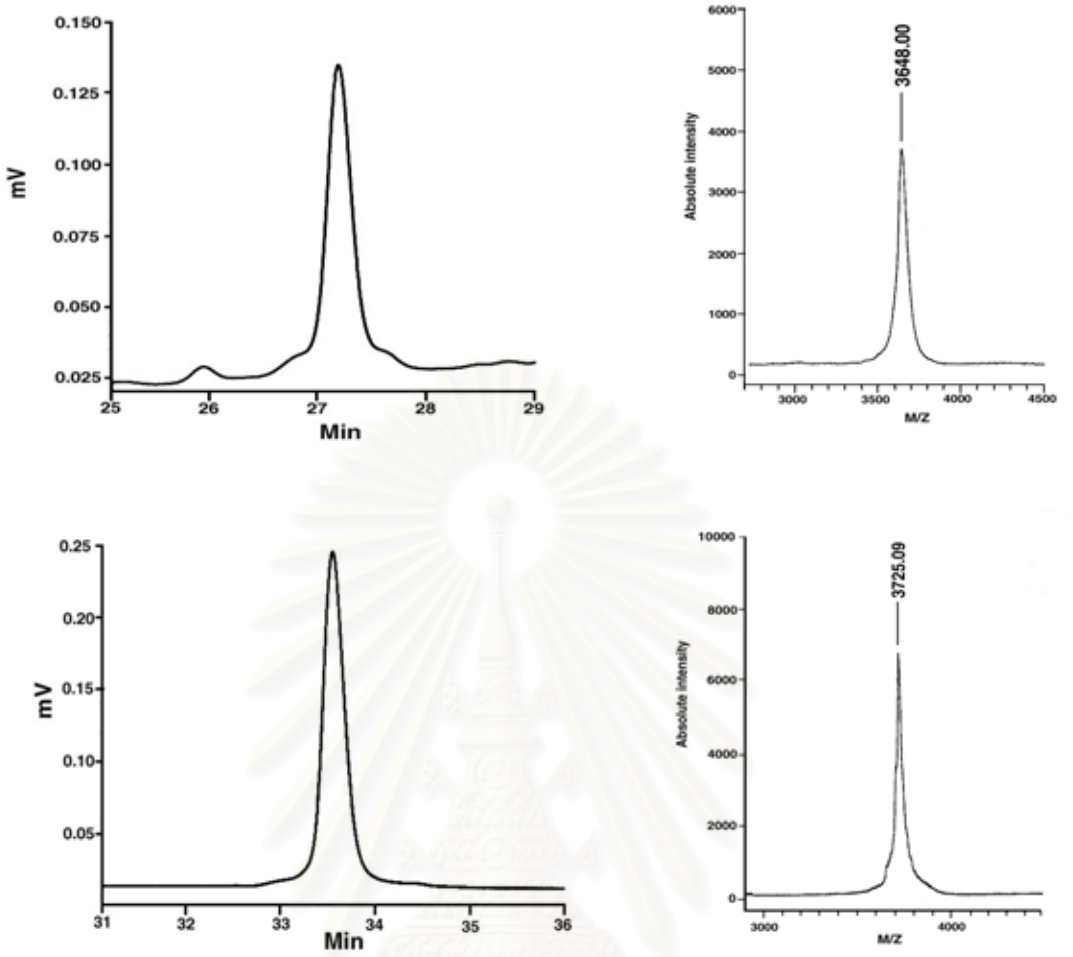


Figure 5.5 Chromatogram and MALDI-TOF MS analysis of purified peptide recognized by monoclonal antibody SG813



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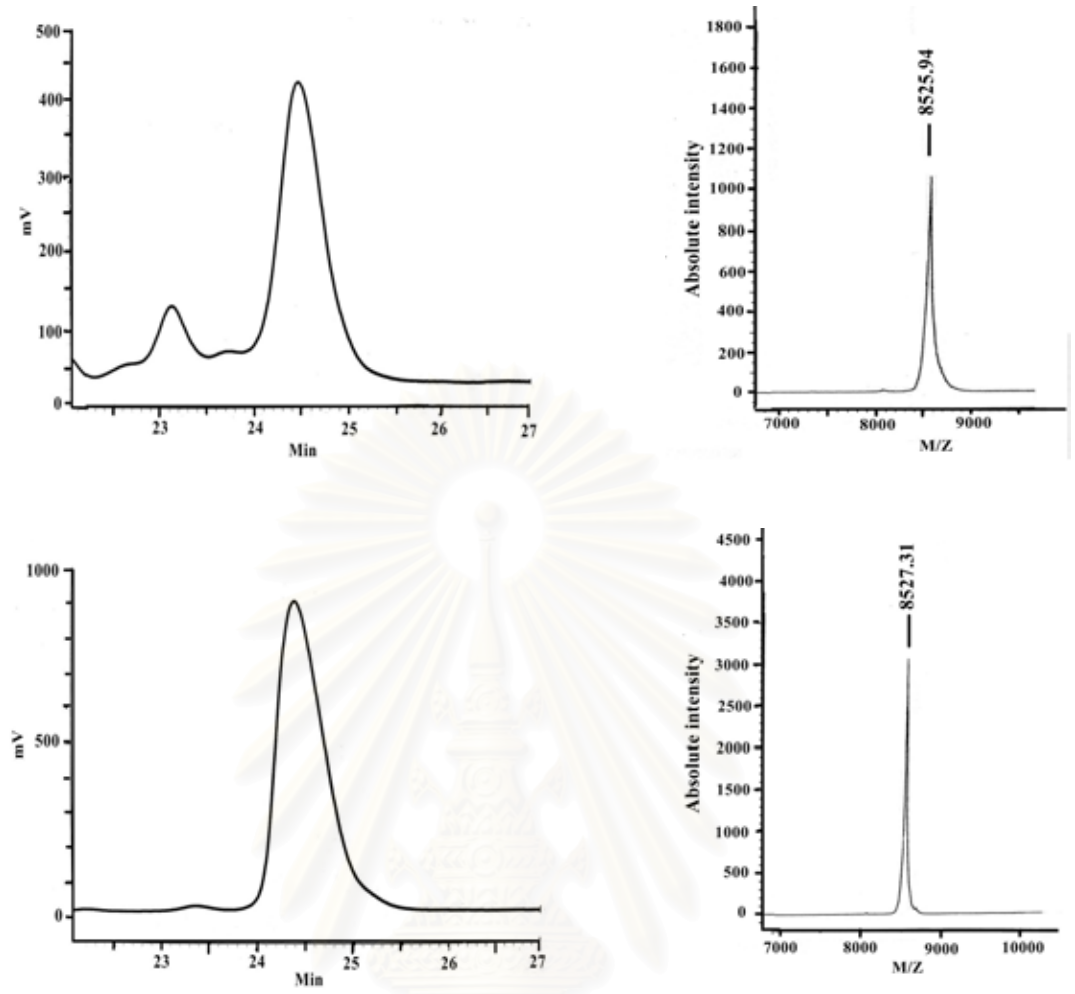

Figure 5.6 Chromatogram and MALDI-TOF MS analysis of purified peptide recognized by monoclonal antibody SG843

```
SG813-4                                VNLQYPE  RETVSELAAQ
Pej     MRSIAVVVLL  VVMALSLQGT  VADSSLKYFE  REVVSELAAQ
Pev1    MARCFVVLAF  LALAAMSLQV  ATAQDDLKYF  EPEVVAELAA
Pev2    MMRSAVVVAL  LMVAMSLQLT  AAQEDLKFE   REVVAELAAQ


SG813-4 ILRVAQGPSA  FVA
Pej     ILRVAQGPSA  FVAGPHKRNS  ELINSLLGIP  KVMTDAGRR
Pev 1   ILRVAQGPSA  FVAGPHKRNS  ELINSLLGIP  KVMNDAGRR
Pev 2   ILRVAQGPSA  FVAGPHKRNS  ELINSLLGIP  KVMNDAGRR
```

Figure 5.7 Amino acid sequence of the peptide SG813-4 isolated by SG 813 monoclonal antibody and compared with the prepro PDH from Pej; *P. japonicus* (Ohira et al., 2002) and Pev; *P. vannamei* (Desmoucelles-Carette et al., 1996). Underlined residues are the different amino acid compositions.

5.4 Discussion

We were able to isolate eight hybridoma clones producing antibodies against various sinus gland peptides in the eyestalk of *P. monodon*. The yield of hybridoma from five fusions were quite low. This reflected the low number of positive antibodies obtained in this experiment. As such, it reduced the opportunity to obtain other antibodies specific to unknown and/or known peptides such as MIH (Krungkasem et al., Genbank: accession numberBAB69830) CMG peptide (Udomkit et al., 2000) FMRFamide peptide family (Sithigorngul et al., 2002a) and NPY/PP family (Sithigorngul et al., 2002b). Therefore, increasing the yield of hybridoma production by any means would increase the opportunity to obtain antibodies against rare peptides.

Characterization of monoclonal antibodies by immunohistochemistry in the eyestalk and dot-ELISA of sinus gland peptide fraction separated by one step of RP-HPLC demonstrated that all recognized sinus gland and different number of neurons in medulla terminalis X-organ complex (MTXO) in eyestalk of *P. monodon* (Fig 5.1). With dot-ELISA, most of the monoclonal antibodied showed low affinity to sinus gland extract fractions after one step purification except for SG706, SG813 and SG 843 monoclonal antibodies that have sufficient affinity to monitor the to isolated sinus gland peptides during purification. One of the purified peptides recognized by SG813 monoclonal antibody peptide, revealed that the N-terminal sequence of the first 30 residues displayed similarity to part of the PDH-precursor related peptides in *P. japonicus and P. vannamei* (Desmoucelles-Carete et al., 1996; Ohira et al., 2002) (Fig. 5.7).

Interestingly, the classes and subclasses of monoclonal antibodies are IgM but it can still be used to monitor the peptides during purification and the specific peptides were isolate. IgM normally occurs during primary response of the immune system. It is possible that most of the peptides may presence at a very minute amount, therefore the activation of B-cell response may occur late at the last boost.

CHAPTER VI

Summary

1. Allatostatin (AST)-like immunoreactivity was localized in various regions of the eyestalk of *Penaeus monodon* as demonstrated by immunohistochemistry using four anti-AST antibodies.

2. Putative AST receptor in the eyestalk of *P. monodon* was demonstrated in the eyestalk of *P. monodon* including sinus gland using the anti *Drosophila* AST receptor (Dar-2) antiserum. To our knowledge this is the first demonstration of a neuropeptide receptor localized to the crustacean sinus gland. This suggested that ASTs may function directly on the sinus gland as a neuromodulator.

3. Purification and sequencing of AST-like peptides are required in order to study the role of AST in physiological control of normal function.

4. Random monoclonal antibodies against neuropeptide were generated from mice immunized with sinus gland dissected from paraffin section of the eyestalk *P. monodon*. Four monoclonal antibodies were isolated. All of these monoclonal antibodies bound to sinus gland and neurons in eyestalk and bound to putative peptides in different fractions of eyestalk extract separated by RP-HPLC.

5. The antibodies were used to monitor the presence of the peptides during purification of sinus gland extracted by RP-HPLC using dot-ELISA method. CHHs (Sgp I, III and IV) were isolated and identified with SG 24, 26 and 293 monoclonal antibodies, respectively.

6. An unknown peptide with molecular mass of 9127.56 Da 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 peptide previously reported.

7. Other monoclonal antibodies against various sinus gland neuropeptides were obtained and partially characterized. All of these antibodies bound to sinus gland and neurons in the eyestalk of *P. monodon*. SG 813 monoclonal antibody recognized peptide with molecular mass range from 3500-3700 Da. One of these (molecular mass 3648.00, SG 813-4) were subjected to sequence analysis. The N-terminal sequence of the first 30 residues revealed similarity to part of PDH-precursor related peptides in *P. japonicus and P. vannamei*.

8. Two purified peptides recognized by SG 843 monoclonal antibody were obtained and had molecular mass of 8525.94 and 8527.31 Da.

9. Sequencing of other purified peptides is required in order to increase basic knowledge of physiological control in *P. monodon*.

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APPENDICES

APPENDIX A

BUFFER AND REAGENT PREPARATION

1. Phosphate buffered saline (PBS) 0.15 M pH 7.2

NaCl	8.00	g
KCl	0.20	g
KH_2PO_4	0.20	g
Na_2HPO_4	1.15	g
or $Na_2HPO_4 . 7H_2O$	2.15	g
Distilled water adjust volume to	1,000.0	ml

2. Bradford solution (Bradford. 1976)

Coomassie brilliant blue G-250	100.0	mg
Methanol	50.0	ml
85% Phosphoric acid	100.0	ml
Distilled water adjust volume to	1,000.0	ml

3. 1% Merthiolate

Thimerosal (Sigma)	1.0	g
Distilled water adjust volume to	100.0	ml

4. 5% Blotto

Skim milk	5.0	g
1% Merthiolate	1.0	ml
Triton X-100	0.1	ml
PBS adjust volume to	100.0	ml

5. Davidson's fixative

95 % Ethyl alcohol	300.0	ml
40 % Formaldehyde	100.0	ml
Glacial acetic acid	100.0	ml
Distilled water	300.0	ml

6. Gelatin coating solution.

Chrome alum	0.05	g
Gelatin	1.0	g
Distilled water	100.0	ml

Dissolved chrome alum in distilled water followed by gelatin and warmed up the temperature to 50-70°C for gelatin melting. Filtered with filter paper no. 1. Kept the filtrate in 60°C until use. To coat the slide, glass slide were washed clearly with distilled water. Dipped the slide once into the warmed gelatin coating solution then air-dried and kept in slide box until use.

7. Modified Alsever's solution (anticoaggulant)

27 mM	Sodium citrate	7.94	g
336 mM	Sodium chloride	19.64	g
9 mM	EDTA	3.35	g
Distilled water adjust volume to		1000.0	ml

APPENDIX B

REAGENT PREPARATION FOR HYBRIDOMA PRODUCTION

1. RPMI medium

RPMI 1640 (Roswell Park Memorial Institute - Gibco BRL, USA)

	10.4	g
D-glucose (Sigma)	3.6	g
L-glutamine (Sigma)	0.2923	g
Sodium pyruvate ($C_3H_3O_3Na$) (Sigma)	1.1005	g
$NaHCO_3$	2.0160	g
HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Sigma)		
	5.9525	g
H_2O (Milli Q water)	1,000.0	ml

The solution of penicillin G, streptomycin and kanamycin were added to the final concentration of 20,000 units, 200 mg and 200 mg per liter, respectively. The medium was sterilized by millipore membrane (pore size 0.22 μm) filtered and stored at 4°C.

2. RPMI medium with serum

RPMI medium (1)	80.0	ml
Fetal calf serum (FCS, Starrate, Australia)	20.0	ml
or Calf bovine serum (CBS, Starrate, Australia)		
100 X HT supplement (Gibco BRL, USA)	1.0	ml
-10 mM sodium hypoxanthine		
-1.6 mM thymidine		

3. Hybridoma selective medium (HAT medium)

RPMI medium (1)	80.0	ml
FCS	20.0	ml
HT supplement	1.0	ml
50 X Aminopterin (Sigma)	2.0	ml
1 % Mouse red blood cell		

4. Fusion solution (40 % polyethylene glycol)

Polyethylene glycol (PEG)	4.0	g

The solution was prepared by dissolving 4.0 g of polyethylene glycol in 6 ml of RPMI medium (1). The solution was incubated at 37 ^{o}C in CO_2 incubator before use.

5. Freezing medium (12 % DMSO)

Dimethylsulfoxide (DMSO, Sigma)	12.0	ml
RPMI medium (2.1)	88.0	ml

The medium was stored in 4 ^{o}C before use.

APPENDIX C

BUFFER AND SOLUTION FOR SDS-PAGE AND WESTERN BLOT ANALYSIS

1. Stock solution :

1.1 Monomer solution (30 % T, 2.7 % C_{Bis})

Acrylamide (BIO-RAD)	58.4	g
Bis (N,N'-methylene-bis-acrylamide, BIO-RAD)	1.6	g
Distilled water adjust volume to	200.0	ml

Stored at 4^{o}C in the dark bottle.

1.2 4 X Running gel buffer (1.5 M tris-Cl pH 8.8)

Tris (hydroxymethyl) aminomethane (BIO-RAD)	36.3	g
Distilled water adjust volume to	200.0	ml

adjusted pH with HCl

1.3 4 X Stacking gel buffer (0.5 M tris-Cl pH 6.8)

Tris	3.0	g
Distilled water adjust volume to	50.0	ml

adjusted pH with HCl

1.4 10 % SDS

SDS (sodium dodecyl sulfate, BIO-RAD)	50.0	g
Distilled water adjust volume to	500.0	ml

1.5 10 % Ammonium persulfate (freshly prepared)

Ammonium persulfate (BIO-RAD)	0.1	g
Distilled water	1.0	ml

1.6 Running gel overlay (0.375 M tris-Cl pH 8.8, 0.1 % SDS)

1.5 M Tris (3.1.2)	25.0	ml
10 % SDS (3.1.4)	1.0	ml
Distilled water adjust to	100.0	ml

1.7 2 X Treatment buffer (0.125 M tris-Cl pH 6.8, 4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol)

0.5 M Tris (3.1.3)	2.5	ml
10 % SDS (3.1.4)	4.0	ml
Glycerol	2.0	ml
2-Mercaptoethanol	1.0	ml
Distilled water	0.5	ml

2 Preparation of separating gel and stacking gel

2.1 Stacking gel for SDS-PAGE 4 % gel (4 % T 2.7 % C_{Bis})

Monomer solution (3.1.1)	2.66	ml
0.5 M tris-Cl pH 6.8 (3.1.3)	5.0	ml
10 % SDS (3.1.4)	0.2	ml
Distilled water	12.2	ml
10 % Ammonium persulfate (3.1.5)	100.0	µl
TEMED	10.0	µl

2.2 Separating gel for SDS-PAGE 7.5 % gel (7.5 % T 2.7 % C_{Bis})

Monomer solution (3.1.1)	7.5	ml
1.5 M tris-Cl pH 8.8 (3.1.2)	7.5	ml
10 % SDS (3.1.4)	0.3	ml
Distilled water	14.55	ml
10 % Ammonium persulfate (3.1.5)	150.0	µl
TEMED	20.0	µl

Table 1D Preparation of separating gel and stacking gel for SDS-PAGE

	Separating gel		Stacking gel
	7.5 % T 2.7 % C_{Bis}	10 % T 2.7 % C_{Bis}	4 % T 2.7 % C_{Bis}
30 % T 2.7 % C_{Bis} (1.1)	2.5 ml	3.35 ml	0.67 ml
1.5 M tris-Cl pH 8.8 (1.2)	2.5 ml	2.5 ml	-
0.5 M tris-Cl pH 6.8 (1.3)	-	-	1.25 ml
10 % SDS (1.4)	0.1 ml	0.1 ml	0.05 ml
Distilled water	4.85 ml	4.2 ml	3.05 ml
Mixed and deaerated using vacuum pump			
10 % Ammonium persulfate (1.5)	50 µl	50 µl	25 µl
TEMED	3.5 µl	3.5 µl	2.5 µl
Mixed and rapidly poured between the glass plate			

3. Runing buffer :

3.1 SDS-PAGE Tank buffer

(0.025 M tris pH 8.3, 0.192 M glycine, 0.1 % SDS)

Tris	12.0	g
Glycine	57.6	g
10% SDS (3.1.4)	40.0	ml
Distilled water	4,000.0	ml

4. Staining and destaining solution

4.1 Staining solution for protein (Coomassie blue)

4.1.1 Stain stock (1% Coomassie blue R-250)

1% Coomassie blue R-250	1.0	g
Distilled water	100.0	ml

4.1.2 Stain (0.1 % Coomassie blue R-250, 50% methanol, 10% acetic acid)

Stain stock (4.1.1)	50.0	ml
Methanol	250.0	ml
Acetic acid	50.0	ml
Distilled water	500.0	ml

4.2 Destaining solution for Coomassie blue

4.2.1 Destain I (50% methanol, 10% acetic acid)

Methanol	500.0	ml
Acetic acid	100.0	ml
Distilled water	1,000.0	ml

4.2.2 Destain II (5% methanol, 7% acetic acid)

Methanol	50.0	ml
Acetic acid	70.0	ml
Distilled water	1,000.0	ml

Method of protein staining

A gel was stained with 0.1 % Coomassie blue R-250, 50% methanol, 10% acetic acid for 5-6 hrs. The gel was then washed in destain I for 1 hr. with 1-2 changes and followed by destain II until the gel was cleared. After washing in distilled H_2O for a few times, the gel was dried in a gel air dryer (BIO-RAD)

6 Towbin transfer buffer pH 8.8

6.1 25 mM tris, 192 mM glycine, 20 % methanol pH 8.8

The buffer consisted of the following ingredients :

Tris	3.03	g
Glycine	14.4	g
Methanol	200.0	ml
Distilled water adjusted to	1,000.0	ml

The buffer was pre-chill before use

APPENDIX D

REAGENT FOR DETERMINATION OF ISOTYPES AND SUBISOTYPES OF MONOCLONAL ANTIBODIES

Hybridoma sub-isotyping kit, mouse (Zymed) contents :

A) Rabbit anti-Mouse IgG1 (γ_1 chain specific)

B) Rabbit anti-Mouse IgG2a (γ_{2a} chain specific)

C) Rabbit anti-Mouse IgG2b (γ_{2b} chain specific)

D) Rabbit anti-Mouse IgG3 (γ_3 chain specific)

E) Rabbit anti-Mouse IgA (α chain specific)

F) Rabbit anti-Mouse IgM (μ chain specific)

G) Rabbit anti-Mouse kappa light chain

H) Rabbit anti-Mouse lambda light chain

I) Normal Rabbit Serum, (Negative Control)

J) Positive Control, Monoclonal Mouse IgG1(Mouse IgG1 is in RPMI-1640 with 10 % FBS)

K) Substrate Buffer, Concentration (10 X) (1 M citrate, pH 4.2, containing 0.03 % H_2O_2)

L) ABTS Substrate, Concentrated (50 X) (2,2-azino-di[3-ethylbenzthiazoline sulfonic acid])

M) Blocking Solution, Concentration (50 X) (25 % BSA in PBS and 0.05 % NaN_3)

N) HRP-Goat anti-Rabbit IgG (H+L), Concentrated (50 X)

O) Goat anti-Mouse IgGAM, Concentrated (50 X) (0.5 mg/ml in PBS containing 10 % glycerol and 0.05 % NaN_3)

BIOGRAPHY

Miss. Nanthika Panchan was born on February 22, 1974 in Bangkok. She graduated with a Bachelor Degree of Science (Biochemistry and Biochemistry technology) from Chiangmai University, Chiangmai in 1996 and Master Degree of Science (Biotechnology) from Chulalongkorn University, Bangkok in 1999.

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Academic publication :

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