THESIS

PURIFICATION AND CHARACTERIZATION OF ANDROGENIC GLAND HORMONE FROM GIANT FRESHWATER PRAWN, *Macrobrachium rosenbergii* de Man

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GRADUATE SCHOOL, KASETSART UNIVERSITY

2008

THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Master of Science (Biochemistry)

DEGREE

Biochemistry	Biochemistry
FIELD	**DEPARTMENT**

TITLE: Purification and Characterization of Androgenic Gland Hormone from Giant Freshwater Prawn, *Macrobrachium rosenbergii* de Man

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THESIS

PURIFICATION AND CHARACTERIZATION OF ANDROGENIC GLAND HORMONE FROM GIANT FRESHWATER PRAWN, *Macrobrachium rosenbergii* de Man

WARANGKANA NARKSEN

A Thesis Submitted in Partial Fulfilment of
the Requirements for Degree of
Master of Science (Biochemistry)
Graduate School, Kasetsart University
2008

Warangkana Narksen 2008: Purification and Characterization of Androgenic Gland Hormone from Giant Freshwater Prawn, *Macrobrachium rosenbergii* de Man. Master of Science (Biochemistry), Major Field: Biochemistry, Department of Biochemistry. Thesis Advisor: Associate Professor Sunanta Ratanapo, Ph.D. 165 pages.

Androgenic gland (AG) in higher crustacean is responsible for the development of male characteristics. Implantation of the whole androgenic gland into giant freshwater prawn (*Macrobrachium rosenbergii* de Man) led to transformation of female recipient into male. The purposes of this research were to purify, characterize and clone the AGH from androgenic tissues of the freshwater prawn. The androgenic tissues and vas deferens were homogenized in acid-alcohol followed by acetone precipitation. AGH found specifically in the androgenic tissues was purified by the reverse phase HPLC on Sep-Pak C18 or Lichrosorb column. Analysis by MALDI-TOF MS indicated that the purified AGH was a protein with molecular mass of 10.2 kDa. No change of mass after deglycosylation by PNGase F or reduction of disulfide bridges by DTT revealed that the purified AGH had no glycosylation and disulfide bridges. Peptide mass fingerprint of the purified AGH did not show matching to any androgenic gland hormones available in the NCBI database, indicating that the purified AGH differs from the numerous crustacean androgenic gland hormones.

Partial cDNA fragment of AGH was amplified using degenerated primers, designed based on the nucleotide sequences of AGHs from other crustacean species. No nucleotide sequences of these PCR products were similar to androgenic gland hormone of isopods and insulin-like androgenic gland factor of *C. quadricarinatus*. The 766 5′ ESTs of *M. rosenbergii* androgenic gland from cDNA library were established and categorized into seven categories on the basis of general functions. The SMART domain search tool predicted the existence of a domain between 66-105 residues of F-D010 clone similar to insulin growth factor-binding protein and to insulin / insulin-like growth factor / relaxin family as same as the pro-*Cq-IAG* ,three pro-AGHs in isopods.

The biological activities of *M. rosenbergii* AGH fractions from each purification step were determined by observation of masculinization after direct injection of each sample into the post-larvae female freshwater prawns. The result was not successful because of high mortality of the prawns. However, *in vivo* biological activity assay of this protein should be further investigated to confirm the being of androgenic gland hormone of the purified protein.

Warangkana Narksen
Student's signature

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29 / 5 / 08

ACKNOWLEDGEMENTS

I would like to express my deep appreciation to my advisor, Associated Prof. Sunanta Ratanapo; and all the committees, Assistant Prof. Amornrat Promboon, Dr. Sittiruk Roytrakul, Assistant Prof. Lertluk Ngernsiri and Mr. Wigrom Rungsin, for their guidance, encouragement and continuous support through the course of this work.

I am especially indebted to Dr. Kazuei Mita, Insect Genome Research Unit, National Institute of Agrobiological Science, Tsukuba, Ibaraki JAPAN. I am very grateful to Ms. Nuanjun Wichukchinda and Ms. Wimala Inunchot for their constant help in cloning techniques and nucleotide sequencing. I would also like to extend my appreciation to the members of Proteomics Service of BIOTEC for technical assistant in the proteomics works.

I must thank all the members of the Department of Biochemistry, Faculty of Science, Kasetsart University, including the teachers, staffs and students. The friendship, enlightening discussions, and the overall atmosphere center spirit have made my stay at this department pleasant and enjoyable. Special thanks go to my friends, Bee, Boon, Bum, Gade, Jib, Kit, Mon, Na, Name, Nok, Opal, Ple, Pueng, Sai, Ta, Ying (Nipaporn), and Ying (Sirinapa). I am very happy to be here with all of you.

Finally, this work is dedicated to my dear parents and my sister for their love and encouragement.

This work was supported by grants from Kasetsart University Research and Development Institute (KURDI) 2005 and 2007, TRF-Master research grants (2006), and Research Grants for Master Degree student from the Graduate School, Kasetsart University (2006).

Warangkana Narksen

April 2008

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LIST OF ABBREVIATIONS

bp	base pair
^{o}C	degree Celsius
Da	Dalton
dNTP	deoxynucleotide-5-triphosphate
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetraacetic acid
EtBr	ethidium bromide
et al.	et. Alli (Latin), others
Fig.	Figure
g	gram
g	gravity force
Gal	galactose
h	hour
IPTG	isopropyl thiogalactoside
kb	kilobase pair
l	litre
LB	Lulia-Bertani medium
M	Molar
mg	milligram
min	minute
ml	mililitre
mM	milimolar
MOPS	3-(N-morpholino) propane sulfonic acid
MW	molecular weight
ng	nanogram
O.D.	optical density
PCR	polymerase chain reaction
rpm	revolution per minute

LIST OF ABBREVIATIONS (Continued)

RNA	ribonucleic acid
RNase	ribonuclease
s	second
SDS	sodium dodecyl sulphate
TAE	Tris acetate EDTA electrophoresis buffer
TE	Tris EDTA
Tm	melting temperature
Tris	Tris (hydroxymethyl) aminomethane
U	unit
UV	ultraviolet
V	volt
w/v	weight by volume
X-gal	5-bromo-4-chloro-3-indolyl--D-galactopyranosid

PURIFICATION AND CHARACTERIZATION OF ANDROGENIC GLAND HORMONE FROM GIANT FRESHWATER PRAWN, *Macrobrachium rosenbergii* de Man

INTRODUCTION

Aquaculture plays an important role in Thailand with a very high portion of total export products and national income. In the last two decades, the technology of intensive farming has expanded significantly with freshwater and marine farming. The giant freshwater prawn (*Macrobrachium rosenbergii*) is also one export commodity with a high inclination in the world market. In 2004, Thailand has exported this prawn up to 3,942.6 ton with the US$ 20.1 million, which is increased in high production.

Males of the giant freshwater prawn *M. rosenbergii* grow faster and reach higher weights at harvest than females since females use considerable energy in egg production. This is a fact which makes the culture of all-male populations desirable. It has thus become obvious that an efficient biotechnology for producing all-male prawn populations is required, especially in countries in which economically valuable crustaceans constitute an important source of income.

The androgenic gland is considered to be the exclusive organ that produces the androgenic gland hormone which induces male sexual development and inhibits the female differentiation in crustacean (Hasegawa *et al.*, 1993; Sagi *et al.*, 1997). In freshwater prawn, androgenic gland removal from immature males resulted in sex reversal, with complete female differentiation. Similarly, androgenic gland implantations into immature females lead to the development of the male reproductive system. Sex-reversed *M. rosenbergii* animals were capable of mating with normal specimens to produce offspring. Many evidences in *M. rosenbergii* support the idea of a proteinaceous androgenic gland hormone (King, 1964; Sagi, 1988; Awari and Kiran, 1999; Sun *et al.*, 2000). Unlike the androgenic gland hormone of isopods, the exact

nature of the androgenic gland hormone of decapod Crustacea has not yet been identified.

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To transform these females to male prawns, high production of androgenic gland hormone by molecular technique and injection it into those female prawns would be a more practical way to increase the prawn production than grafting of androgenic gland. The aims of this research are to purify, characterize and clone the androgenic gland hormone in giant freshwater prawn.

OBJECTIVES

1. To purify and characterize the molecular structure of androgenic gland hormone from the androgenic gland of giant freshwater prawn.

2. To clone and determine the nucleotide sequence of the full-length androgenic gland hormone gene of giant freshwater prawn

3. To study the biological activity of purified androgenic gland hormone.

LITERATURE REVIEW

1. Biology of giant freshwater prawn

The giant freshwater prawn (*Macrobrachium rosenbergii* de Man) is also known as the giant river prawn or the Malaysian prawn. This species is commercially important for its value as a food source.

1.1 Nomenclature

All the freshwater prawns that have been cultured belong to the genus *Macrobrachium*, the largest genus of the family Palaemonidae. About 200 species have been described, almost all of which live in freshwater at least for part of their life.

The giant freshwater prawn, *M. rosenbergii*, was one of the first species to become scientifically known, the first appearing in 1705. In the past, the nomenclature of freshwater prawns had been very confusing. Generic names have included Cancer (Astacus) and Palaemon. Previous names of *M. rosenbergii* have included *Palaemon carcinus*, *P. dacqueti*, and *P. rosenbergii*. In 1959, its present scientific name, *Macrobrachium rosenbergii* (De Man, 1879) was universally used.

1.2 Taxonomy

Some taxonomists recognize a western subspecies (found in the waters of the east coast of India, Bay of Bengal, Gulf of Thailand, Malaysia, and the Indonesian regions of Sumatra, Java and Kalimantan) and an eastern subspecies (inhabiting the Philippines, the Indonesian regions of Sulawesi and Irian Jaya, Papua New Guinea and northern Australia). These are referred to as *Macrobrachium rosenbergii dacqueti* (Sunier, 1925) for the western form and *Macrobrachium rosenbergii rosenbergii* (De Man, 1879) for the eastern form. However, from the perspective of freshwater prawn

farmers, exact nomenclature has little relevance, especially because the species *M. rosenbergii* has been transferred within its natural geographical range and been introduced into many other zones where it may become established.

Kingdom: Animalia
Phylum: Arthropoda
Subphylum: Crustacea
Class: Malacostraca
Order: Decapoda
Suborder: Pleocyemata
Infraorder: Caridea
Family: Palaemonidae
Genus: *Macrobrachium*
Species: *Macrobrachium rosenbergii*

1.3 Distribution

There are about 200 species of *Macrobrachium* in the world, of which 49 are commercial. Twenty-seven of the commercial species are found in Asia and the Pacific. Most live in freshwater. A few species live in brackishwater in the mouths of rivers. Relationship of the globally distributed freshwater prawn genus is shown as a phylogenetic tree. (Fig. 1)

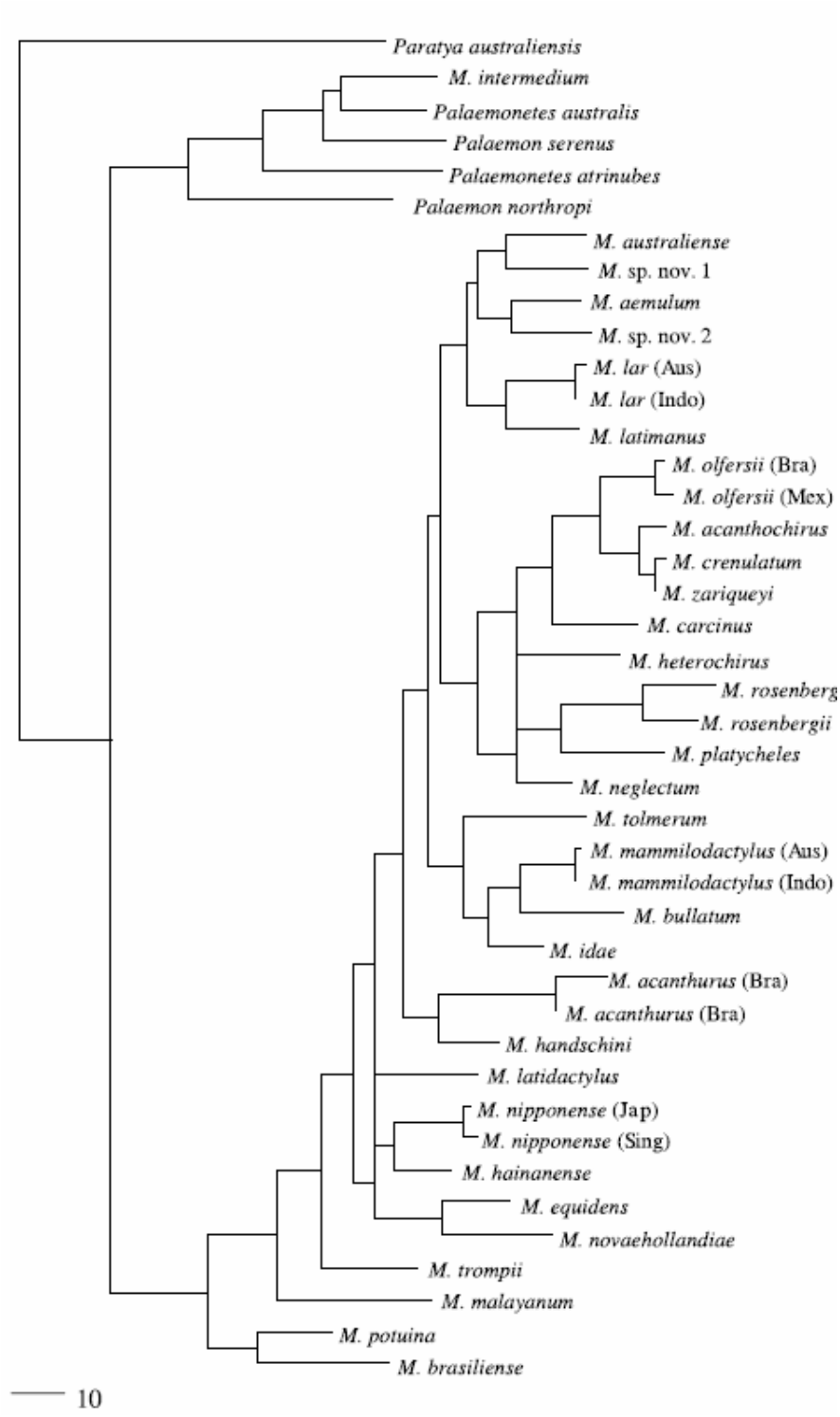

Figure 1 Phylogenetic relationships of the globally distributed freshwater prawn genus

Source: Murphy and Austin, 2005

M. rosenbergii is found extensively in the tropical and subtropical waters of the Indo- Pacific region in Malaysia, Thailand, the Philippines, India, Shri Lanka, Bangladesh, Myanmar, Indonesia and Vietnam. They are generally found in freshwater, in ponds, rivers, lakes, ditches, canals, depressions, low-lying floodplains and river mouths. Most of the species spend their early life in brackishwater that is connected directly or indirectly with the sea. Some species complete their life cycle in freshwater. Prawns move upstream, entering lakes and even paddy fields, up to about 200 kilometres from the sea. This type of migration is observed not only in *M. rosenbergii* but also in other species of *Macrobrachium*.

M. rosenbergii can be reared at temperatures ranging from 14 to 35°C. For growth, the optimal temperature and optimal pH range is 29-31°C and 7.0-8.5, respectively (Diaz-Herrera and Buckle-Ramirez. 1993).

1.4 Life cycle

Like other crustaceans, giant freshwater prawns have a hard outer shell that must be shed regularly in order to grow. This process is called “molting”. Because of these periodic molts, growth occurs in increments, rather than continuously. This results in four distinct phases in the life cycle; egg, larvae, postlarvae (juvenile), and adult (Fig. 2.). The number of molts and the durations of intermolts are not fixed, and depend on the environment, particularly temperature and the availability of food.

In the natural environment, mating of *M. rosenbergii* takes place all year round, although, due to environmental reasons, peak mating takes place only during certain periods of the year. A female prawn, with matured gonad, copulates just after molting with a male prawn having a hard shell. During copulation, the male deposits a gelatinous mass, or spermataphore, on the underside of the thorax of the female, between the walking legs. The female prawn releases its eggs a few hours to a few days after copulation. The number of eggs depends on the size of the female. A fully

matured female of 50-100 g can carry 50,000-100,000 eggs. But at first maturity, due to the female's small size, it lays only 5000-20,000 eggs.

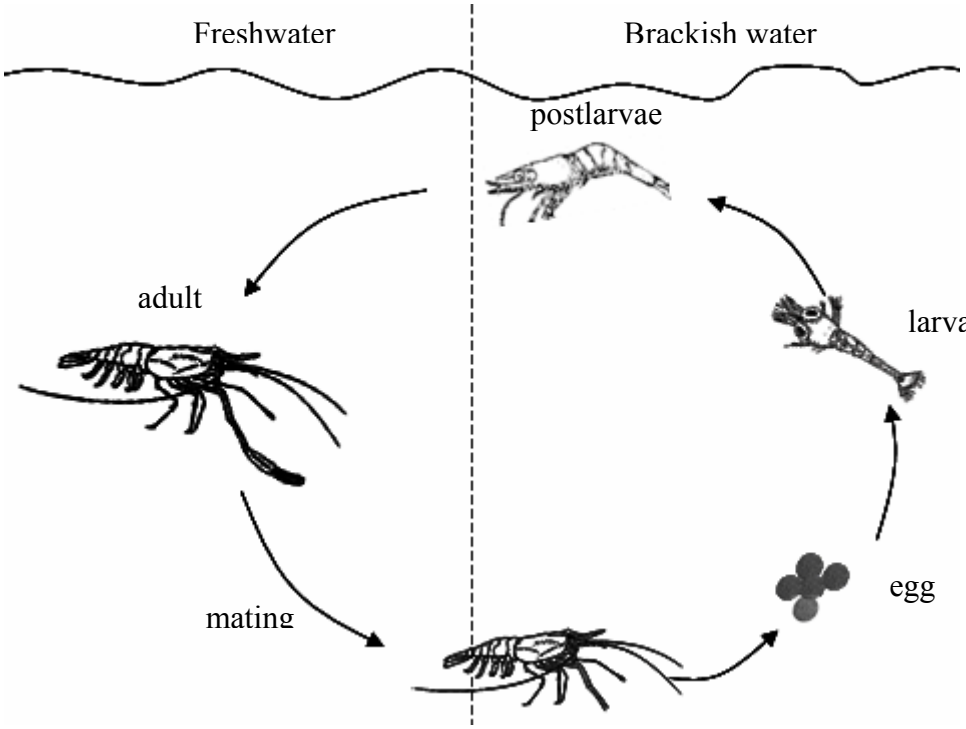

Figure 2 The life cycle of *M. rosenbergii*.

Giant freshwater prawn eggs of this species are slightly elliptical, with a long axis of 0.6-0.7 mm, and are bright orange in color until 2-3 days before hatching when they become grey-black. This color change occurs as the embryos utilize their food reserves. Eggs are incubated for 21 days and then hatch.

Larvae hatch during the night. Rapid movement of the female pleopods disperses the newly hatched larvae, which normally swim with their heads down and 'jump' when they contact a surface. Larvae need brackish water to survive at this stage. Even if larvae hatch in freshwater, they will not survive if they are not put into within two or three days. Larvae in the wild generally eat zooplankton, small insects and larvae of other aquatic invertebrates.

Larvae in a hatchery take a minimum of 26 days to metamorphose into postlarvae. Most scientists accept that the larvae go through 11 distinct stages (Uno and Kwon, 1969) before metamorphosis, each with several distinguishing features as decribed in table 1. and illustrated in Fig. 3. However, from stage VI onwards their size is variable, which has led to some researcher to describe only eight stages (Ling, 1969). Stage 1 larvae (zoeae) are just under 2 mm long (from the tip of the rostrum to the tip of the telson). Larvae swim upside down by using their thoracic appendages and are positively attracted to light. By stage XI they are about 7.7 mm long. Newly metamorphosed postlarvae are also about 7.7 mm long and are characterized by the fact that they move and swim in the same way as adult prawns. They are generally translucent and have a light orange-pink head area.

Table 1 Several distinguishing features in eleven distinct larvae stages of *M. rosenbergii* before metamorphosis

Larvae stage	Age (days)	Distinguishing features
I	1	Sessile eyes
II	2	Stalked eyes
III	3-4	Uropods present
IV	4-6	Two dorsal teeth
V	5-8	Telson narrows and elongated
VI	7-10	Pleopod buds present
VII	11-17	Pleopods biramous
VIII	13-20	Pleopods with setae
IX	15-22	Endopods of pleopods with appendices internae
X	17-23	3-4 dorsal teeth on rostrum
XI	23-35	Teeth on half of upper dorsal margin
postlarvae	23-35	Adult behavior

Source: Nandlal and Pickering (2005)

Postlarvae can tolerate a wide range of salinity, but freshwater is their normal habitat. Two to three weeks after metamorphosis, the PL move against the current and head towards freshwater canals and rivers. They abandon the planktonic habit at this stage and become omnivorous, feeding on aquatic insects and their larvae, phytoplankton, seeds of cereals, fruit, small mollusca and crustacean, fish flesh, slaughterhouse waste and animal remains. They move by crawling and generally swim with their dorsal side uppermost. They can swim rapidly.

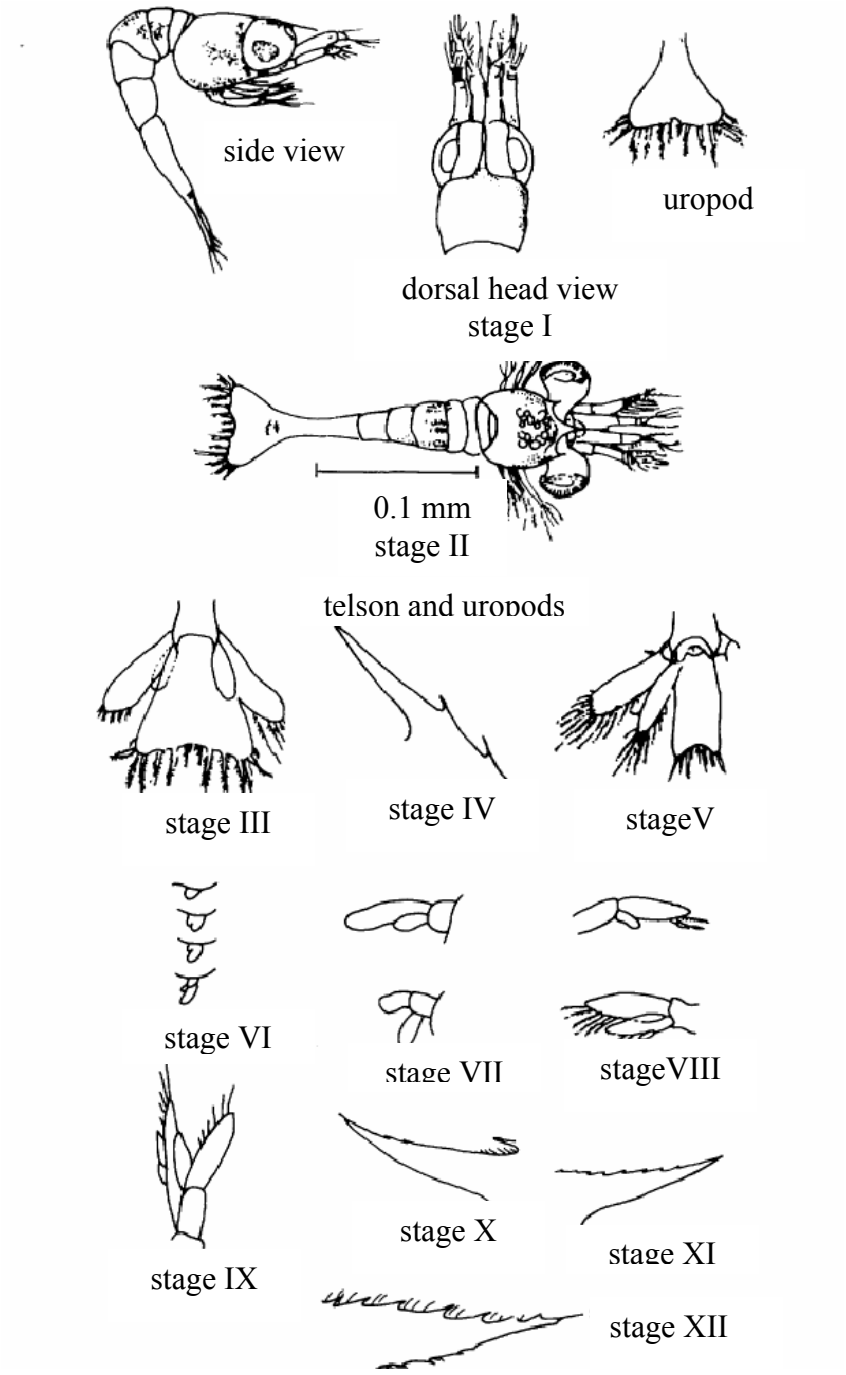

Figure 3. Several distinguishing features in eleven distinct larvae stages of *M. rosenbergii* before metamorphosis

Source: Uno and Kwon (1969)

1.5 Morphology of mature *M. rosenbergii*

M. rosenbergii is an invertebrate with exoskeleton or shell. The giant freshwater prawns (defrosted shell-on tails) looks a lot like the giant tiger prawn (*Penaeus monodon*), but they are bigger, chunkier, lighter in color, and their shells are always on. In fact, it is a species of freshwater shrimp (not prawn).

The body of postlarvae and adult prawns is devided into two parts that consists of the cephalothorax known as the cephalon (head) and the abdomen (tail). The bodies of giant freshwater prawns are divided into 20 segments known as somites. There are 14 segments in the head, which are fused together and invisible under a large dorsal and lateral shield, known as the carapace. The carapace is hard and smooth, except for two antenna on either side. The first antenna is just below the eye socket and the second antenna (the hepatic antenna) is lower down and behind the first antenna. The carapace ends at the front in a long beak or rostrum, which is slender and curved upwards. The rostrum extends further forward than the antennal scale and has 11-14 teeth on the top and 8-10 underneath (Fig. 4).

The front portion of the cephalothorax has six segments and includes the eyes and five pairs of appendages. The final three of these six segments can be seen if the animal is turned upside down and the appendages of the thorax are moved aside. The front portion of the cephalothorax has 6 segments that are stalked eyes, first antenna, second antenna, mandibles (used to grind food), first maxilla (transfer food into the mouth) and second maxilla (beat water to promote the respiratory function).

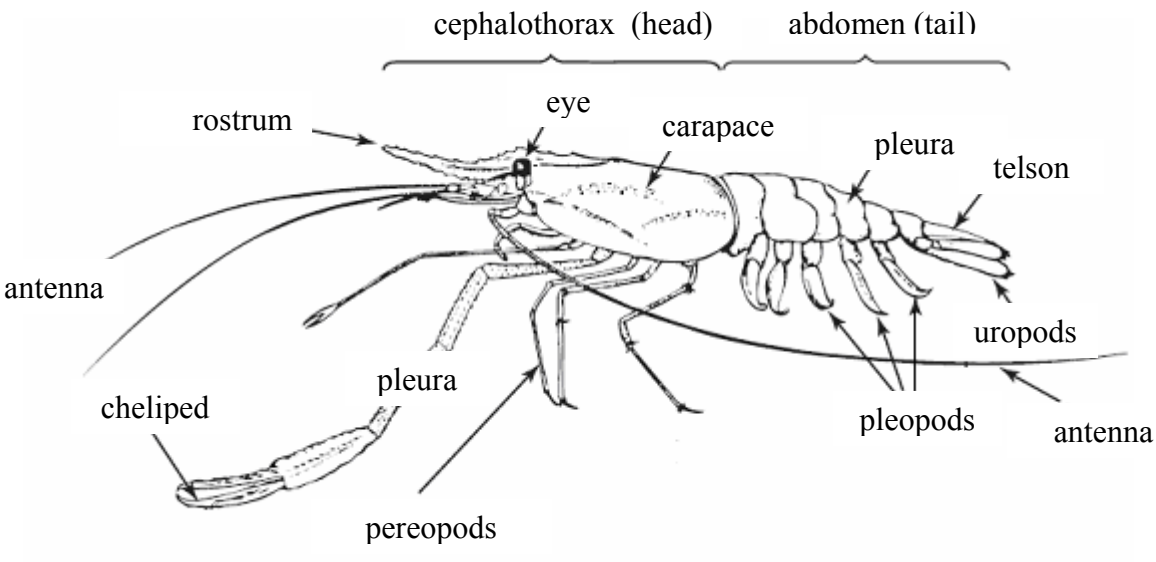

Figure 4 The external morphology of male *M. rosenbergii*
Source: New (2002)

The rear portion of the cephalothorax, known as the thorax, consists of 8 fused segments which have easily visible pairs of appendages. These appendages consist of 3 sets of maxillipeds and 5 pairs of pereopods. The first and second maxillipeds are similar to the first and second maxilla and function as mouthparts. The third maxillipeds are also mouthparts but look rather like leg. The first and second pereopods have pincers (chela). These pincer-ended legs are also called chelipeds. The first pereopods are slender but the second pair bear numerous small spines and are much stronger than any other pereopods. The second chelipeds are used for capturing food, as well as in mating and fighting behavior. The left and right second pereopods (chelipeds) of *M. rosenbergii* are equal in size, unlike some other *Macrobrachium spp*. In adult males they become extremely long and reach well beyond the tip of the rostrum. The third, fourth and fifth pereopods, which are much shorter than the second cheliped, have simple claws (not pincers), and are sometimes called walking legs. Eggs are extruded from oval gonopores in the base of the third pereopods of females, which are covered with a membrane. In males, sperm is extruded from gonopores which are covered by flaps, situated in the base of the fifth pereopods. (Fig. 5)

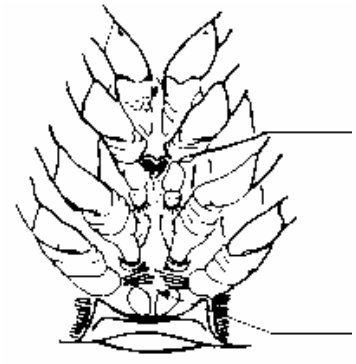

Figure 5 The situation of male and female gonopore of *M. rosenbergii*

The abdomen (tail) is very clearly divided into 6 segments, each bearing a pair of appendages known as pleopods or swimming legs. The first five pairs of swimming legs are soft. In females they have attachment sites for holding clusters of eggs within the brood chamber. In males, the second pair of swimming legs is modified for use in copulation. This spinous projection is known as the appendix masculina (Fig. 6). The sixth pair of swimming legs, known as uropods, are stiff and hard. The telson is a central appendage on the last segment and has a broad point with two small spines which protude further behind the point. The telson and the uropods form the tail fan, which can be used to move the prawn suddenly backwards.

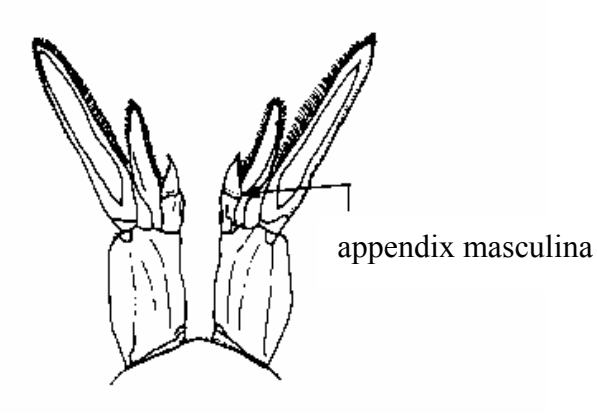

Figure 6 The appendix masculina in male *M. rosenbergii* at the second pair of pleopods

M. rosenbergii can be distinguished from other species in the genus by the some characteristics. It has a very long rostrum, with 11-14 dorsal teeth and 8-10 ventral teeth. The tip of its telson reaches distinctly beyond the posterior spines of the telson. The second chelipeds of adult male is longer and thicker than in other species and have blunt spines. The movable finger of the second chelipeds of the adult male is covered by a dense velvet-like fur (except the extreme tip) but this fur is absent from the fixed finger and the rest of the cheliped.

From the morphology described above, it is can be concluded that male and female *M. rosenbergii* can be distinguished by external characteristics. It is the largest known of all *Macrobrachium spp.*, adult males having been reported with a total body length of up to 33 cm, and adult females of up to 29 cm. Mature *M. rosenbergii* males are bigger than the females, with their cephalothorax larger and their second pair of thoracic legs comparatively longer and thicker. The cephalothorax of the male is also proportionately larger and the abdomen narrower than the female's. In male, the second pair of pereopods is quite long and has many spines while the second pair of female is not so long and is spineless. The male genital pore is situated at the base of the fifth pereopod but female genital pores are situated at the bases of the third pereopods. The abdominal pleura of the female are comparatively longer and the abdomen wider. The orange- colored maturing gonad is easily visible. In immature males, there is a raised hard point on the first segment of the abdominal part of the body (Fig. 7).

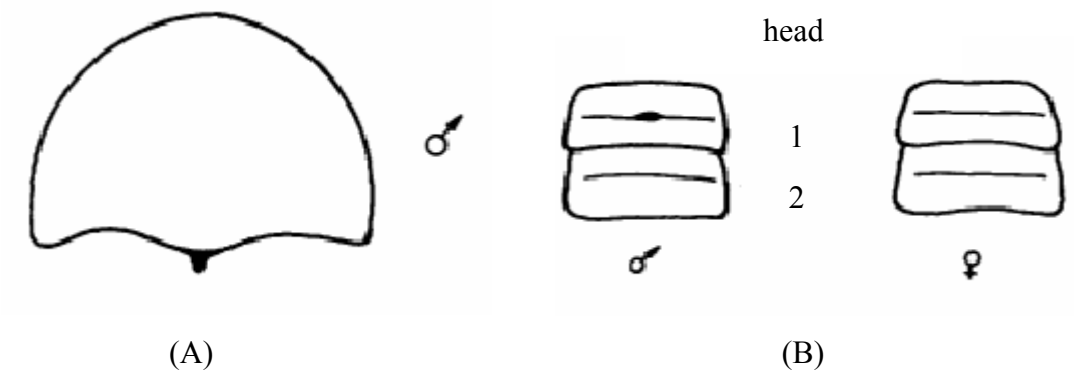

Figure 7 Medium cross section of first abdominal segments of male *M. rosenbergii* shows the hard point position (A). The ventral side of first abdominal segments in male ane female (B).

Source: New and Singholka (1985)

The internal reproductive structures of *M. rosenbergii* are located in the cephalothorax. In males, they consist primarily of pairs of testis which are fused and lie mid-dorsally in the cephalothorax, giving rise to the vas deferens. The paired vas deferens are simple tubes that end in terminal ampulla, which contain the spermatophores and open at the gonopores on the coxae of fifth pereopods. During mating, the ampullae extrude the spermatophors, containing the sperm mass. Besides these structures, an androgenic gland is attached to the vas deferens. In females, the paired ovaries are located dorsally to the stomach and hepatopancreas. They give rise to a pair of oviducts which extend towards, and open into, the gonopores on the basal segment of the third pereopods.

The difference between prawn and shrimp can be distinguished by external morphology. The plate of the second abdominal segment of shrimp overlaps both in front and behind while all of the abdominal side plates of prawn overlap like tiles from the front (Fig. 8).

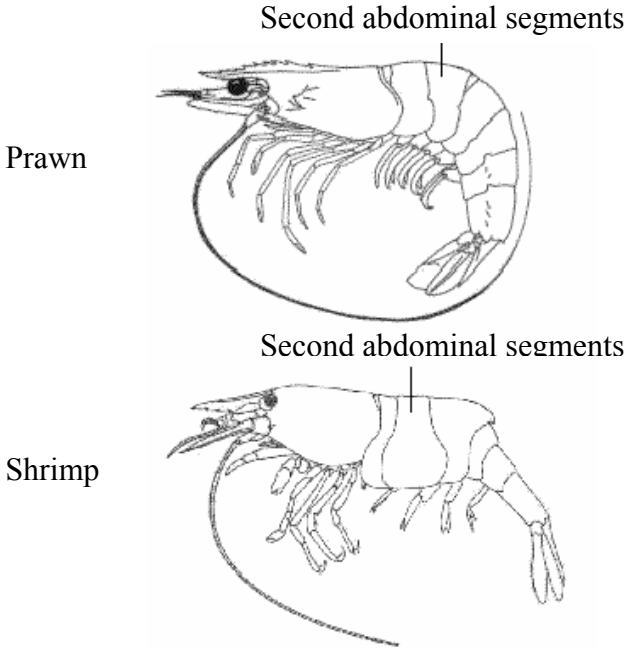

Figure 8 The difference of the plates of the second abdominal segments between prawn and shrimp

1.6 Morphotypic Differentiation of Males of the *M. rosenbergii*

Due to differences in climate, weather and natural environment, adult males of the fresh-water prawn *M. rosenbergii* in a single-age population can be categorized into three morphotypes. Each morphotype represents a different stage in the development of the adult males from small males (SM) through orange-claw (OC) males to blue-claw (BC) males (Cohen *et al*., 1981). BC male grows to a large size. The ratio of claw to body length is 1.6±0.1. The male is territorial, its breeding behavior is complex and growth is comparatively slow. OC male is a little bigger than the median size of the blue claw variety and has orange-colored claws. The ratio of claw to body length is 1.0±0.05. The rate of fertilization of eggs is comparatively slow, but growth is fast. SM male is the smallest of all the subspecies with spineless claws. The ratio of claw to body length is 0.5±0.1. At the time of copulation, these adopt the ‘snake’ mating strategy. Their growth is the slowest of the three varieties.

All males are capable of developing through all of the above stages, but individual males largely differ in the rate in which they transform from one morphotype to another. It was observed that SM and BC males are more sexually active than OC males, while the OC males grow more rapidly than SM and BC males (Ra'anan and Sagi, 1985). The development of the reproductive system (testes, sperm ducts, and ampullae), as an indication of sexual activity, was compared with the development of the midgut gland (hepatopancreas), an organ which is suggested to be in correlation with somatic growth. The hepatopancreas is significantly larger and the reproductive system is relatively smaller, in relation to body size, in OC males. The opposite is true of SM and BC males, in which the reproductive system is significantly larger while the hepatopancreas is much smaller in relation to body size. Thus, the relative sizes of the two organs of an individual prawn are closely associated with its position in the male developmental pathway from the SM through OC to the BC morphotype (Sagi and Ra'anan, 1988).

2. Androgenic gland hormone

Among invertebrates Crustacea Malacostraca constitute the first group of animals in which the existence of sex hormones was demonstrated. In the amphipod *Orchestia gammarella*, Charniaux-Cotton first reported that the androgenic glands (AG) are responsible for male differentiation and development (Cronin, 1947). Later, androgenic glands were identified in a variety of species from nearly all orders of Malacostracans (higher crustacean). This androgenic gland hormone (AGH) controls male differentiation and other physiological processes such as pigmentation and behavior. Androgenic gland hormone is essential for spermatogenesis. The differentiation of copulating brushes on walking legs and of male appendages on the abdominal legs is also triggered by AGH.

2.1 The localization and histological structure of androgenic gland

Androgenic glands are generally observed as cords of epithelial cells, folded upon themselves, and covered in connective tissue, with cells showing oval or

round nuclei and often vacuoles (Charniaux-Cotton, 1960). Standard histological techniques revealed androgenic glands consisting of cords of epithelial cells attached to the posterior vas deferens, either appearing as multiple cords of cells, a single cord of cells, or a cord associated with masses of cells. Each androgenic gland was sheathed in connective tissue, which attached the androgenic gland to the vas deferens. The androgenic gland in *A. vulgare* is located at the tip of each of the three pairs of testes. To determine the distribution of AGH in the male reproductive system, an immunohistochemical study using antibodies against different components of the androgenic gland prohormone molecule of *A. vulgare*, showed strong immunoreactivity to all three of these antibodies, while the testis, the seminal vesicle, and the vas deferens did not show immunostaining (Hasegawa *et al*., 2002).

The observation of the secretory mechanism of the AGH in *A. vulgare*, the secretory granules containing AGH are transferred to the extracellular space through the intercellular canaliculi particularly developed for exporting the peptide hormone. (Hasegawa and Nakajima, 2001)

Androgenic glands with similar structures have been observed in various species of higher crustaceans. The cords of androgenic gland tissue attached to the posterior vas deferens of the crayfish *Procambarus blandingii*, *P. viae-viridis*, *Cambarus bartonni*, *C. diogenes* (Puckett, 1964) and *Cherax destructor* (Warnock, 1975; Fowler and Leonard, 1999). SEM examination revealed that the androgenic glands were covered in connective tissue, and that extensions of the connective tissue attach the glands to the vas deferens. Connective tissue has been implicated in the attachment of androgenic glands in prawns (Hoffman, 1969) and crabs (Joshi and Khanna, 1987; Minagawa *et al*., 1994). The cellular structure of the androgenic glands of *C. destructor* is similar to that of other decapod crustaceans, consisting of epithelial cells with large oval nuclei, and often showing heavy vacuolation (Carlisle, 1959; Hoffman, 1969; Thampy and John, 1972; Fingerman, 1987).

In *M. rosenbergii*, the histological examination and transmission electron microscopy of the structure of the androgenic gland was reported by Veith and

Malecha (1983). The androgenic gland appears as strands of cells in a pyramidal cluster loosely associated with the terminal vas deferens. The individual cell strands are surrounded by a thin layer of connective tissue (Fig. 9). At least three cell types were observed in the androgenic gland. The population of these cell types vary significantly among three morphotypes with the BC morphotype having the highest cell density. The cells are generally polygonal, measuring from 5–12 μm by 15–25 μm. The most striking difference among the cells of the three morphotypes was found in the organization of the rough endoplasmic reticulum (RER). In the OC, the RER is fairly abundant, slightly swollen and unorganized; whereas in the BC, the RER is most abundant, highly organized and formed into stacks in some areas. (Sun *et al*., 2000) The correlation of androgenic gland cell structure to spermatogenic activity and morphotypic differentiation histologically in *M. rosenbergii* was examined. Ultrastructure of the androgenic gland was compared among the molt stages, but distinct histological changes were not observed in relation to spermatogenesis during the molt cycle. On the other hand, among the three morphotypes, the androgenic gland was largest in the blue-claw males, containing developed rough endoplasmic reticulum in the cytoplasm. (Okumura, 2004)

2.2 The biological role of androgenic gland hormone

In malacostracan crustaceans, male sexual differentiation is controlled by AGH produced by a male-specific organ, the androgenic gland. According to Cronin (1947), the testes of the decapod crustacean, the blue crab *Callinectes sapidus*, were attached by a layer of epithelium and a layer of connective fibers. Later, glandular tissue attached to the vas deferens was observed in the crayfish *Cambarus montanus* (Faxon, 1884). Since the discovery of androgenic gland by Charniaux-Cotton (1954) in the amphipod *O. gammarella*, the results of androgenic glands implantation experiments revealed the involvement of this gland in the regulation of male differentiation and spermatogenesis in this amphipod as same as in the terrestrial isopod *Armadillidium vulgare* (Legrand, 1955). This latter species has subsequently become the main focus of research on the androgenic gland in crustaceans because of the difficulty in measuring AGH activity in other species (Katakura, 1984; Katakura

and Hasegawa, 1983; Charniaux-Cotton and Payen, 1985; Hasegawa *et al.*, 1987, 1991; Suzuki and Yamasaki, 1998). The implantation of androgenic glands into a young female induced masculinization in the recipient. AGH induced the gonadal masculinization and the formation of male appendages on the abdominal legs and caused the differentiation of copulation brushes on walking legs (Legrand *et al*., 1968; Katakura, 1961; Katakura and Hasegawa, 1983; Hasegawa *et al*., 1993; Suzuki and Yamasaki, 1997). It was found to masculinize both primary and secondary sexual characteristics in *A. vulgare* (Legrand, 1955; Katakura, 1961). Suzuki and Yamasaki (1998) found that the first morphological evidence of masculinization after the implantation of a fresh androgenic gland into a young female *A. vulgare* was the development of male-like first endopodites. Repeated implantation of ethanol-treated androgenic glands into young females at different stages of sexual development caused gonad sex reversal at critical stages (Suzuki and Yamasaki, 1998). Since such glands are not active, this experiment was able to show the exact dose and time at which the hormone exerts its masculinizing effect. Moreover, Suzuki (1999) had focused on reversal of female gonads by the AGH during the sexually undifferentiated period through post-embryonic development in *A. vulgare*. The ethanol-treated androgenic gland implantation into each genetic female at various developmental stages to induce sex reversal suggested that AGH is a sex reversing factor that can turn a female gonad into a male gonad. AGH cannot be a sex-determining factor in female *A. vulgare*, as undifferentiated gonads of genetic females are not sex reversed by the hormone.

Based on heterospecific implantations of androgenic gland, Martin and Juchault (1999) showed that the male hormone of terrestrial isopods had a relatively narrow species-specificity, since the hormone of one species was effective in the other species of the same genus. AGH of one species may be potent in another species from a different genus and the reciprocal grafting may be without effect. There is a case with *Porcellio scaber* androgenic gland tested in two different genus, *Armadillidium* and *Porcellionides*, where *P. scaber* androgenic gland implantation induced partial masculinization of *A. vulgare* and complete masculinization of *P. pruinosus*, and whereas the reciprocal implantations had no effect (Martin and Juchault, 1999).

The morphological and physiological effects of the gland have been also described in decapod crustaceans. Touir (1977) described the effects of the androgenic gland on both primary and secondary male characteristics in a number of gonochoristic and hermaphroditic decapod crustaceans. A clear effect on an external characteristic that could serve as a bioassay was described in the shrimp *Leander serratus*. Removal of the androgenic gland, accompanied by amputation of the first pleopods, resulted in the regeneration of undifferentiated pleopods. These results suggest that the AGH is essential for the development and maintenance of such secondary male characteristics. The role of the androgenic gland in *Procambarus clarkia* was studied. Sexually immature females were implanted with androgenic glands. Surviving females containing living androgenic gland implants developed first abdominal appendages that were masculine. In masculinized females which became mature, vitellogenesis was inhibited. Development of many oocytes in the ovaries was inhibited (Taketomi and Nishikawa, 1996). Moreover, the study on the relationships among development of the testis, secondary sexual characteristics and the androgenic gland in *P. clarkii* suggested androgenic gland control the differentiation, development of the testes, and secondary sexual characteristics(Taketomi and Koga, 1996). On the other hand, a study on the organogenesis and differentiation of the genital organs and the androgenic gland in the prawn *Penaeus japonicus* put in question the role of the androgenic gland in the early differentiation of the gonads (Nakamura, 1992; Nakamura *et al*., 1992). Experiments on androgenic gland manipulation in the crayfish *Cherax quadricarinatus* confirmed the role of the gland in regulating the development of male secondary sexual characteristics and in the function of the gonads. Intersex specimens of *C. quadricarinatus*, in which one half has an androgenic gland, develop a male reproductive system on the same side. The contralateral half, which lacks the androgenic gland, has a female reproductive system in a permanently arrested state. The secondary external characteristics of such intersex animals are male-like on both sides (Sagi *et al*., 1997). Androgenic gland ablation in these intersex animals resulted in degeneration of the male reproductive system and in the onset of secondary vitellogenesis in the previously arrested ovarian lobe (Khalaila *et al*., 1999). In such individuals, secondary sexual characteristics, such as the red patch on the propodus, were degenerated and the pleopods were differentiated to

female-like pleopods, complete with feminized ovigerous setae. A quantitative ELISA developed to monitor the onset of secondary vitellogenesis in *C. quadricarinatus* showed a high level of a vitellogenic specific polypeptide in the hemolymph of androgenic-gland-ablated intersex individuals. A low level of the vitellogenic-specific polypeptide was found in the hemolymph of androgenic-gland-implanted females (Sagi *et al*., 1999). Androgenic gland implantation into females affected both primary and secondary sex characters (Khalaila *et al*., 2001), also agonistic behavior of *C. quadricarinatus* implanted females was evaluated by comparing the interactions between males and females. Contests that involved male animals alone were much more intense than those of a male with a female. The aggressive behavior of implanted females was intermediate between that of male/male and male/female with regard to aggressive behavioral features such as the duration of an escalated fight and the duration of grasp (Karplus *et al*., 2000). In another species of crayfish, *C. destructor*, implantation of the androgenic gland into immature females caused inhibition of the development of oosetae and of secondary vitellogenesis (Fowler and Leonard, 1999).

The role of the androgenic gland in male *M. rosenbergii* was studied by various researchers. Bilateral androgenic gland ablation andrectomy in immature males appeared to inhibit male primary and secondary sexual characteristics but induced feminization. Reduced appendix masculina growth rate and mature chelipeds was reduced. Atrophied testes and vas deferens occurred while spermatogenesis degenerated. However, feminization including oogenesis and development of oviducts and female gonopores occurred (Nagamine *et al*., 1980a). In contrast, androgenic gland implantation could musculinize young female recipients. The evidences of masculinization were differentiation of appendix masculina and the development of male gonopores, vas deferens and mature male chelipeds. While male characteristics were developed, vitellogenesis did not complete. (Nagamine *et al*., 1980b). Furthermore, the morphotypic transformation was examined experimentally by bilateral androgenic gland ablation of SM males and OC males. Andrectomy on both small and orange-claw males caused disappearance of the genital papillae and atrophy of the sperm ducts and testes. Moreover, the morphotypic differentiation occurred

(Sagi and Ra'anan, 1988). During spermatogenesis which is related to the molt cycle, the AGH is at rather constant levels and plays a role in maintaining spermatogenesis rather than directly regulating the onset of a specific spermatogenesis stage and that, during the morphotypic differentiation, the androgenic gland is most active in the blue-claw males and plays a role in regulating the observed high mating activity in *M. rosenbergii*. (Okumura and Hara, 2004)

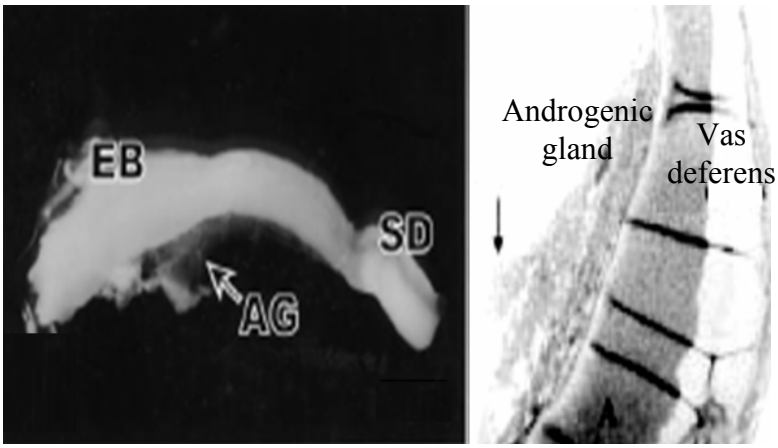

Figure 9 The androgenic gland of *M. rosenbergii* (A) Shown the androgenic gland: AG, sperm duct: SD and ejaculatory bulb:EB (B) Cross-section of the androgenic gland of *M. rosenbergii* shows the location of androgenic gland relative to the terminal vas deferens

Source: Sun *et al.* (2000); Takuji and Hara (2004)

2.3 Molecular structure of androgenic gland hormone

Although the functioning of AGH in Crustacea is similar to that of testosterone in mammals, the chemical nature of AGH is quite different; AGH is not a steroid but can be either a peptide or protein. (Hasegawa, 1987; Martin *et al*., 1999; Okuno *et al*., 1999). Active material was isolated from hypertrophied androgenic glands of *Wolbachia*-parasitised males (Martin *et al*., 1990) or from androgenic glands. The hormone was purified by three steps of reverse-phase high performance

liquid chromatography (HPLC). The fraction thus purified showed a masculinizing activity. Complete amino acid sequencing of the *A. vulgare* AGH showed a structure that seems to be a androgenic gland prohormone in the form of a protein containing three peptide chains, designated A chain, B chain and C peptide. The androgenic hormone precursor (16.5 kDa) was shown to contain the sequence of chains A and B, connected by a C-peptide (45 amino acids).The complex of the AGH precursor is analogous with the proinsulin superfamily peptides (Martin *et al*., 1999; Okuno *et al*., 1999). MALDI-TOF mass spectrometry of the AGH fraction showed a broad peak centered at m/z 11,000 (Martin *et al*., 1999). The amino-acid sequences of the two glycoforms were identical. They consist of two peptide chains, A and B, of 29 and 44 amino acids, respectively, with chain A carrying one N-glycosylated moiety on Asn18. Glycoforms were only differentiated by the size and heterogeneity of the glycan chain. Chain A and chain B each contain a single intrachain disulfide bridge and the two chains are linked by two inter-chain disulfide bridges (Fig. 10). The molecular mass of mature AGH without the carbohydrate moiety was calculated to be 8.7 kDa (Okuno *et al*., 1999).

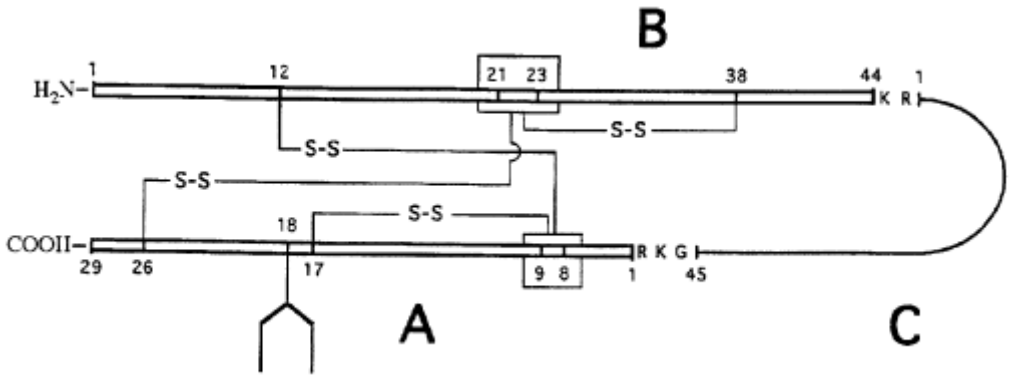

Figure 10 Characterization of androgenic gland hormone in *A. vulgare*
Source: Martin *et al.* (1998)

Based on the amino acid sequencing (Martin *et al*., 1999), Okuno *et al*. (1999) designed two sets of degenerated primers for nested PCR, which resulted in a 317 bp cDNA fragment using the total RNA of the androgenic gland as a template.

After the sequencing of the 317 bp fragments, new AGH specific primers were designed, whose PCR amplification was a 501 bp cDNA. The cDNA included open reading frame encoding 144-amino acid residue proteins. The first 21 amino acid residues were found to be typical of the secretory signal peptide, indicating that AGH is a secretory peptide (Okuno *et al*., 1999). The complete structure of the AGH reported by the group of Nagasawa (Okuno *et al*., 1999) confirmed the structure presented by Martin *et al*. (1999) of chains A, B, and C, the intra- and inter-chain disulfide bridges, and a glycan moiety at Asn18. A molecular probe in a Northern bolt analysis gave the 0.8-kb hybridization signal found exclusively in the RNA preparation from *A. vulgare* androgenic gland (Okuno *et al*., 1999).

Later, there is a case with *Porcellio scaber* androgenic gland tested in two different genus, *Armadillidium* and *Porcellionides*, where *P. scaber* androgenic gland implantation induced partial masculinization of *A. vulgare* and complete masculinization of *P. pruinosus*, and whereas the reciprocal implantations had no effect (Martin and Juchault, 1999). With a polyclonal antibody raised against recombinant precursor of *A. vulgare* androgenic gland expressed by *Escherichia coli*, Hasegawa *et al*. (2002) showed that the androgenic glands of five terrestrial isopods were immunostained and consequently the AGH s of these species shared common epitopes.

Similar AGH s were identified in two other isopod species such as *P. scaber* (Ohira *et al*., 2003; Greve *et al*., 2004) and *P. dilatatus* (Ohira *et al*., 2003), with the amino acid sequence of the mature AGH peptide being highly conserved amongst the three species. Molecular cloning of cDNAs encoding AGH precursors from cDNA fragments encoding AGHs were amplified by RT-PCR using degenerate oligonucleotide primers designed based on the amino acid sequence of *A. vulgare* AGHs. Subsequently, full length cDNAs were obtained by 5'- and 3'-RACE. The AGH cDNA of *P. scaber* and *P. dillatatus* consists of 672 bp with ORF (435 and 438 bp, respectively). The deduced amino acid sequences of *P. scaber* and *P. dillatatus* consists of B chain (44 amino acid), C peptide (45 and 16 amino acids, respectively) and A chain (31 amino acid) and form disulfide bridges in the same manner with *A.*

vulgare. The sequence identities of A and B chain of *P. scaber* and *P. dillatatus* androgenic hormone compared to *A. vulgare* were very high (75-82%).

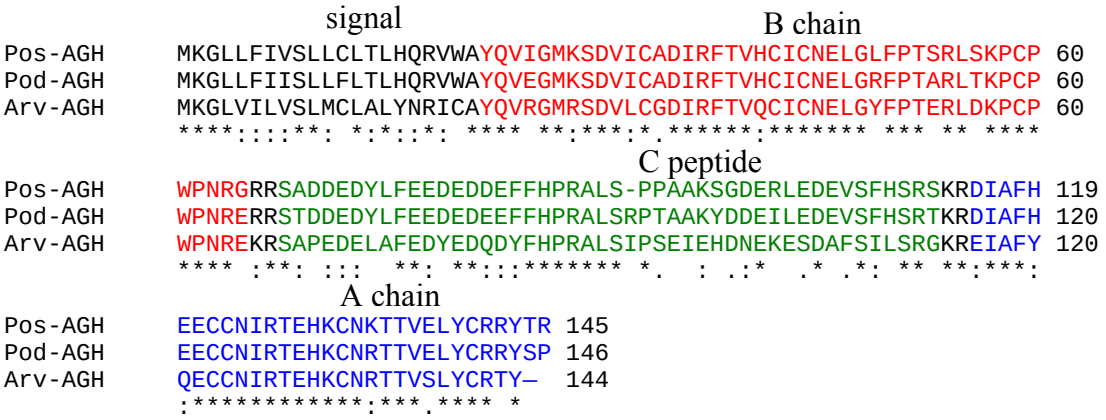

Figure 11 Amino acid sequence alignment of *P. scaber*, *P. dillatatus* and *A.vulgare* androgenic gland hormone precursor (Pos-AGH: *P. scaber*, Pod-AGH: *P. dillatatus*, Arv-AGH: *A. vulgare*)

In decapods, however, no AGH has been thus far identified. The first active molecules suggested to be associated with the androgenic gland in decapod crustaceans were terpenoids and steroids. The terpenoids, farnesylacetone and hexahydrofarnesylacetone, were found in the crab *Carcinus meanas* (Ferezou *et al*., 1978). A steroidogenic nature for the gland was suggested in the freshwater prawn *M. rosenbergii* (Veith and Malecha, 1983). Based on the elucidation of the isopod androgrnic gland hormone and in keeping with some histological findings in decapods (King, 1964; Sagi, 1988) As such, it is surprising that the ultrastructure of the androgenic gland in different crustaceans resembles that of a vertebrate protein-producing cell rather than a steroid-producing cell (King, 1964). This, together with recent histological evidence in prawns supporting the idea of a proteinaceous AGH (Awari and Kiran, 1999; Okumura and Hara, 2004), has given rise to the belief that a proteinaceous androgenic hormone will eventually be purified from the androgenic gland of decapod crustaceans and that it is similar to the *A. vulgare* androgrnic gland hormone (Sun *et al*., 2000; Zhang *et al*., 2000).

Sun *et al*. (2000) studied on total protein analysis of androgenic glands from three male morphotypes of *M. rosenbergii*. Highest protein content was found in the blue-claw morphotype as compared to the other morphotype. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the cell free extract of the androgrnic gland from the three morphotypes revealed four polypeptides (16, 18, 23 and 26 kDa) which quantitatively increase from the sexually immature orange-claw to the sexually mature blue-claw morphotype. They suggested that the 16 and 18 kDa polypeptides could be the AGH.

Cao *et al*. (2006) identified male-specific genes that could be involved in male development in *M. rosenbergii* by screening a subtracted male reproductive tract library and isolated a novel gene named *Mar-Mrr* (*M. rosenbergii* male reproduction-related gene). The *Mar-Mrr* cDNA sequence consists of 683 nucleotides with a 333 nucleotide open reading frame, encoding putative 110 amino acids (11.7473 kDa) precursor protein and a signal peptide consisting of 24 amino acids. Significant developmentally dependent accumulation of the mRNA was observed in the male reproductive tract, specifically in epithelial cells of vas deferens and terminal ampullae. Moreover, *Mar-Mrr* mRNA expression significantly increased during androgenic gland maturation. However, *Mar-Mrr* mRNA was not detected in prawns from the postlarvae stage, when the androgenic gland exhibited little activity.

Manor *et al*. (2007) constructed the subtractive cDNA library from a decapod androgenic gland from the crayfish *Cherex quadricarinatus* and discovered an androgenic gland-specific gene, expressed exclusively in males even at early stages of maturation and termed *Cq-IAG* (*C. quadricarinatus* insulin-like androgenic gland factor) In situ hybridization of *Cq-IAG* confirmed the exclusive localization of its expression to the androgenic gland. Following cloning and complete sequencing of the gene, its cDNA was found to contain 1445 nucleotides encoding a deduced translation product of 176 amino acids. The proposed protein sequence encompasses Cys residue and putative cleaved peptide patterns whose linear and 3D organization are similar to those of members of the insulin/insulin-like growth factor/relaxin family and their receptor recognition surface.(Fig. 12) The identification of *Cq-IAG* is the

first report of a pro-insulin-like gene expressed in a decapod crustacean in a gender-specific manner. However, the androgenic activity study of *Cq-IAG* was not established, *Cq-IAG* is not comfirmed as AGH.

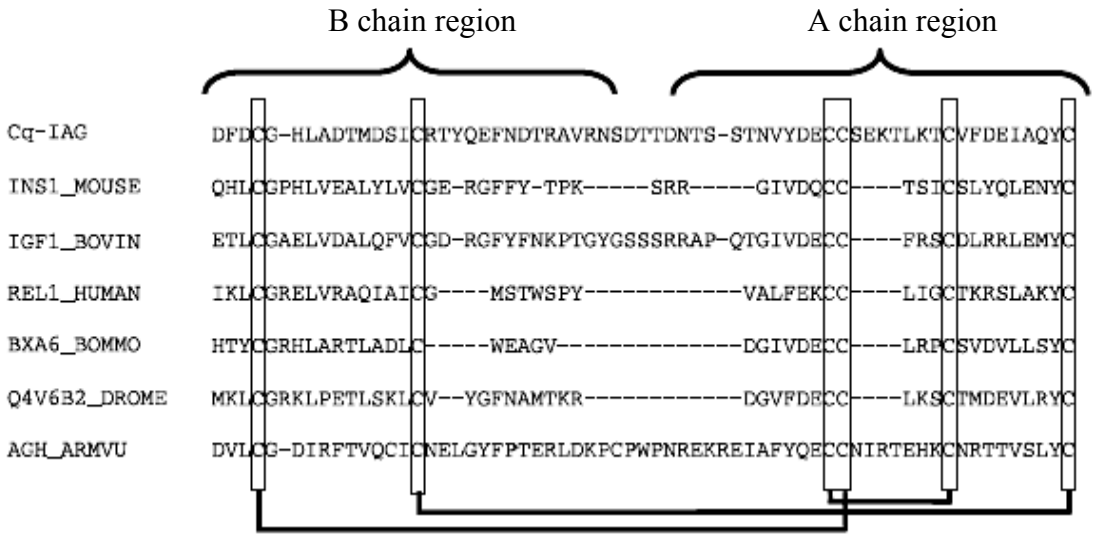

Figure 12 Multiple sequence alignment of the putative mature *Cq-IAG* with representative members of the insulin/insulin-like growth factor/ relaxin family, calculated by ClustalX. The sequences shown include: mouse insulin [INS1_MOUSE, (Wentworth *et al*., 1986)], insulin-like growth \ actor I of bovine [IGF1_BOVIN, (Fotsis *et al*., 1990)], human relaxin H1 [REL1_HUMAN, (Hudson *et al*., 1984)], bombyxin C-1 of *B. mori* [Silk moth, BXA6_BOMMO, (Nagasawa *et al*., 1986)], *D. melanogaster* insulin like protein (Q4V6B2_DROME) and AGH of *A. vulgare* [AGH_ARMVU, (Okuno *et al*., 1999)]. Conserved Cys residues are shown in boxes while the (-)indicates gaps introduced into the amino acid sequence to allow for the maximal degree of identity in the alignment.

Source: Manor *et al*. (2007)

3. Production of all-male populations of the giant freshwater prawn *Macrobrachium rosenbergii*

Differential growth and dominance of larger individuals characterize *M. rosenbergii* populations. Male giant freshwater prawn can grow faster and has a larger size than females, thus male giant freshwater prawn would be economically advantageous. All male culture is the new trend in giant freshwater prawn commercial farming. Nowadays, there are two ways for establishing all-male populations: (1) establishment of monosex cultures through manual segregation and (2) using AG material for producing all-male progeny.

The first production of a male monosex culture of the giant freshwater prawn *M. rosenbergii* by hand segregation in small-scale culture led to the two-times more average weight of the prawns in the all male population campared to all female and mixed populations. Moreover, in all-male culture, the prawns reached market size at a faster rate. The marketing period of fresh product would be prolonged (Sagi *et al*., 1986). Similar results were obtained when a male monosex prawn culture was tested under intensive monoculture conditions in earthen ponds. When the procedure was tested in polyculture ponds, all-male stocking yielded an 18% increase in net income (Hulata *et al*., 1988). It has thus become obvious that an efficient biotechnology for producing all-male prawn populations is required, especially in countries in which economically valuable crustaceans constitute an important source of income (Sagi and Aflalo, 2005) The problem of developing a technology based on this type of manipulation is the difficulty of identifying males at a sufficiently early stage of development. To overcome this problem, all-male offspring production was required.

In *M. rosenbergii*, androgenic gland ablation of immature males resulted in sex reversal with complete female differentiation. Similarly, androgenic gland implantations into immature females lead to the development of the male reproductive system. Obviously, androgenic gland plays an important role in the regulation of male differentiation and in the inhibition of female differentiation. Therefore, androgenic

glands would be an interesting key for producing all-male populations of the giant freshwater prawn.

Due to the ablation of the androgenic gland of *M. rosenbergii* at early stage of development caused sex reversal to females called neofemale. Neofemales are capable of mating and spawning. Sagi and Cohen (1990) based on two crosses showed that the mating of the neofemale with the normal male resulted in 99.1% and 100% male offspring. Aflalo *et al.* (2006) suggested a novel two-step procedure for mass production of all-male populations. First step, postlarvae males were andrectomized resulting with all male progeny at low successful rate. Then, second step, the progeny (presumed males) of neofemales from first step were andrectomized at earlier ages. There was a significant increase in the number of sex reversed animals such as developed ovaries. Finally, mating of neofemales with normal males resulted in all-male offspring. Similary, Rungsin *et al.* (2006) produced all-male stock by neofemale technology in Thai strain of freshwater prawn.

MATERIALS AND METHODS

Materials

1. Prawn samples

Male giant freshwater prawns (*M. rosenbergii*) were collected from Talad-tai market and Kung-ten restaurant, Bangkok. The body weight of an adult giant freshwater prawn was approximately 100 grams.

The prawns were divided into two groups. One was the group which giant freshwater prawns were unilateral eyestalk-ablated and the other was normal (control). One eyestalk of giant freshwater prawns were ablated with sterilized scissors. Immediately, the wound was cauterised by hot soldering iron to stop bleeding. After ablation, the prawns were fed for 2 weeks. Then, their androgenic glands and vas deferens containing androgenic gland were dissected and kept at -80°C prior to use to extract total protein and total RNA.

2. Bacterial strain

2.1 *Echerichia coli* strain JM109

E. coli strain JM109 with genotype: e14-(McrA-) recA1 endA1 gyrA96 thi-1 hsdR17 (rK- mK+) supE44 relA (lac-proAB) [F' traD36 proAB lacIqZ M15] was purchased from UBI, Canada.

2.2 *E. coli* strain Mach1 -T1

E. coli strain Mach1 -T1 with genotype ΔrecA1398 endA1 tonA Φ80ΔlacM15 ΔlacX74 hsdR(rK- mK+). From Invitrogen.

2.3 *E. coli* strain XL1-Blue

E. coli strain XL1-Blue with genotype endA1, gyrA96, hsdR17, lac , recA1, relA1, supE44, thi-1, [F' lacI^{q}Z ΔM15, proAB, Tn10]. From Clontech, USA.

2.4 *E. coli* strain BM25.8

E. coli strain BM25.8 with genotype supE44, thiΔ(lac–proAB) [F'traD36, proAB, lacI^{q}Z ΔM15] λimm434 (kan^{R})P1 (cam ^{R}) hsdR (r_{k12}–m_{k12} –). From Clontech, USA.

3. General chemicals and reagents

Absolute ethanol (Merck, Germany)
Acetic acid glacial (BDH, UK)
Ammonium acetate (Merck, Germany)
β-mercaptoethanol (Sigma, USA)
Bromophenol blue (Merck, Germany)
Calcium chloride (Sigma, USA)
Chloroform (Merck, Germany)
Formaldehyde (BDH, Germany)
Glucose (Sigma, USA)
Glycerol (BDH, UK)
Hydrochloric acid (Merck, Germany)
Isoamyl alcohol (Merck, Germany)
Isopropanol (Merck, Germany)
2-mercaptoethanol (Sigma, USA)
Polyvinylpyrrolidone (Sigma, USA)
Potassium chloride (Sigma, USA)
Sodium acetate (Merck, Germany)
Sodium chloride (Merck, Germany)
Sodium dodecyl sulfate (Merck, Germany)

Sodium hydroxide (BDH, UK)

Xylene cyanol FF (Sigma, USA)

5. Chemicals for bacterial culture

Bacto agar (Difco, USA)

Bacto tryptone (Difco, USA)

Tryptone (Difco, USA)

Yeast extract (Difco, USA)

6. Chemicals and reagents for molecular cloning

Agarose (Sekem)

Ampicillin (Sigma, USA)

5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) (Sigma, USA)

Chloroform (Merck, Germany)

Dextran sulphate (Sigma, USA)

Diethyl pyrocarbohydrate (DEPC)

100 mM dATP, dCTP, dGTP, and dTTP (Promega,USA)

DNA markers : λ DNA, 1 Kb plus DNA ladder and 100 bp ladder (Gibco,USA)

Ethanol, absolute (Merck, Germany)

Ethidium bromide (EtBr) (Sigma, USA)

Ethylene diamine tetraacetic acid, disodium salt dihydrate (EDTA) (Merck, Germany)

Formaldehyde (BDH, UK)

Formamide (BDH, UK)

Isopropyl-β-D-thiogalactoside (IPTG) (Sigma, USA)

Phenol (Sigma, USA)

Tris-(hydroxy methyl)-aminomethane (Sigma, USA)

7. Chemicals and reagents for protein purification and characterization

2-mercaptoethanol (Sigma, USA)
Acetone (Carlo Erba, Italy)
Acetronitrile (Merck, Germany)
Acrylamide (ACROS, USA)
Ammonium bicarbonate (Sigma, USA)
Ammonium persulfate (Merck, Germany)
Bovine serum albumin (Sigma, USA)
Coomassie Brilliant blue G-250 (Bio basic, Canada)
Copper sulfate (Sigma, USA)
Dithiothreitol (Pharmacia, USA)
Ethanol (Carlo Erba, Italy)
Folin–Ciocalteu reagent (Fluka, USA)
Glycerol (APS, Australia)
Hydrochloric acid (Carlo Erba, Italy)
Iodoacetamide (Pharmacia, USA)
Methanol (Merck, Germany)
N, N'-Methylene-bis-acrylamide (Pharmacia, USA)
Phenol red (Sigma, USA)
Protein markers: full length Rainbow™ Molecular Weight Markers (GE healthcare, USA)
Potassium sodium tartrate (Carlo Erba, Italy)
Sodium carbonate (BDH, UK)
Sodium dodecyl sulfate (Biobasic, Canada)
Sodium hydroxide (BDH, UK)
TEMED (Fluka, USA)
Tricine (Acros, USA)
Trifluoroacetic acid (Fluka, USA)
Tris(hydroxymethyl)aminomethane (Biobasic, Canada)

8. Kits for molecular cloning

NucleoSpin Extraction Kit (MACHEREY - NAGEL, Germany)
NucleoTrap gel extraction kit (Clontech, USA)
NucleoTrap mRNA Purification Kits (Clontech, USA)
NucleoSpin Plasmid Extraction Kit (MACHEREY - NAGEL, Germany)
Packagene Lamda DNA packaging system (Promega, USA)
pGEM-T vector Systems (Promega, USA)
RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Germany)
SMART ^{TM} cDNA Library Construction Kit (Clontech, USA)
TRIzol reagent (Gibco, USA)

8. Enzyme and Restriction enzymes for molecular cloning

IMMOLASE DNA polymerase (Bioline, UK)
Proteinase K (Gibco, USA)
Real Taq DNA polymerase (RBC, Taiwan)
Restriction enzymes (BioLabs, USA)
RNase A (Sigma, USA)

9. Enzyme for protein work

Endoproteinase Lys-C (Sigma, USA)
N-glycosidase F (Sigma, Germany)
Trypsin (Sigma, USA)

10. Miscellaneous

0.2 μm Millipore membrane filter (Schleicher & Schuell, Germany)
Liquid nitrogen (TIG)
Minerol oil (Sigma, USA)
PVDF membrane
Whatman 3MM papers

11. General equipments

Autopipette: Pipetteman (Gilson, France)
Balance (Satorious)
Centrifuge, refrigerated centrifuge: Model BR 4i (JOUAN SA)
Centrifuge, microcentrifuge: Model Spectrafuge 16M (Labnet)
Hot plate
Incubator water bath: Model INNOVA 3100 (New Brunswick Scientific)
Magnetic stirrer
Nanodrop spectrometer: Model ND-1000 (Nanodrop, USA)
Microwave
pH meter
UV Visible spectrophotometer (Beckman, USA)
Vortex mixer

12. Equipments for proteomics

Electrophoresis: Hoefer SE260 (Amersham Biosciences, USA)
HPLC: Alliance 2690 with RI and PDA (Waters, USA)
Lichrosorb C18 column
MALDI-TOF mass spectrometer: Reflex IV (BRUKER, Germany)
Sep-Pak Vac C18 cartridge (Waters, Ireland)
Trans-Blot semi-dry system (Bio-Rad).
Q-TOF LC/MS: LTQ (Thermo Fisher Scientific, Germany)

13. Equipments for molecular cloning

Autoclave: Model HA-300M

Submerged Agarose Gel Electrophoresis System: GelMate 200 (Toyobo)

Hot air oven: Model 838F (Fisher Scientific)

Incubator: Model IPR 150.XX2.C

Incubator shaker: Model IOC400.XX2.C (GALLENKAMP PLC)

Laminar flow: BH-120 (GelmanScience)

Thermal cycle: Model 480 (PerkinElmer)

Methods

1. Extraction and purification of androgenic gland hormone

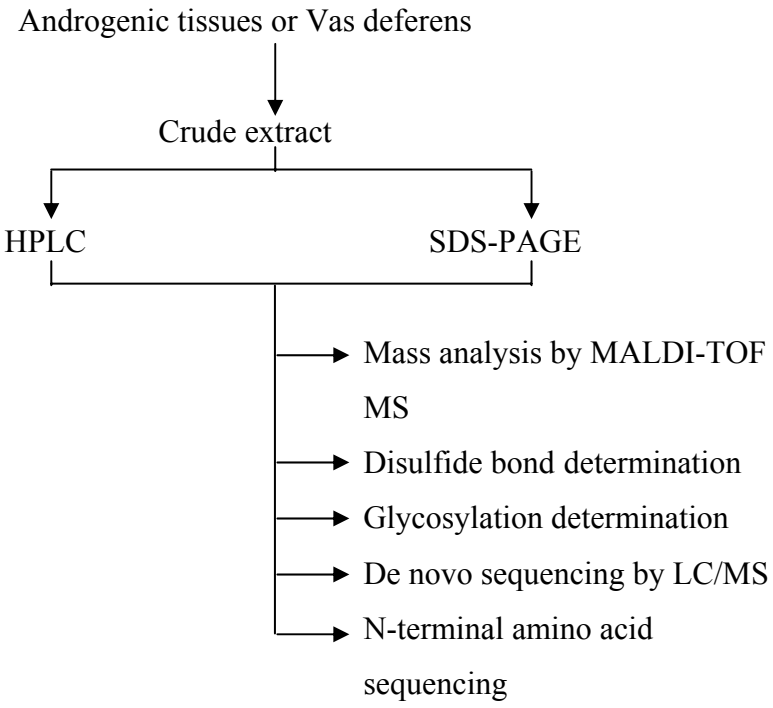

Figure 13 Flow chart of extraction, purification and molecular characterization androgenic gland hormone from androgenic tissues or vas deferens of *Macrobrachium rosenbergii*

1.1 Protein extraction

Crude protein was extracted from the androgenic tissues or vas deferens of freshwater prawns according to the method of Eng *et al.* (1990). Each tissue was homogenized with 10 volumes of acid-alcohol (0.2 M acetic acid in 75% ethanol) and then centrifuged at 12,544 x g for 15 min at 4 ^{o}C. The supernatant was collected and precipitated with 8 volumes of cold acetone. The protein pellet was harvested by centrifugation at 12,544 x g for 15 min at 4 ^{o}C and resuspended in distilled water. The protein concentration of the acetone precipitated sample was determined by Lowry's method (see appendix) using bovine serum albumin as a standard.

1.2 Protein purification

1.2.1 Reverse phase column chromatography by Sep-Pak Vac C_{18} cartridge

Solid-phase extraction is the simplest, most cost effective and versatile method of sample preparation. Utilizing low cost, prepackaged, disposable cartridges containing chromatographic packing, a sample analyte of interest is separated from other species in the sample matrix by loading the sample onto the device and selectively eluting the desired compounds using different solvents. C_{18} silica-based bonded phase with strong hydrophobicity; used to adsorb analytes of even weak hydrophobicity from aqueous solutions.

Each acetone-precipitated sample was dissolved in 1 M acetic acid. The acetic acid fraction was then applied to a Sep-Pak Vac C_{18} cartridge, pre-equilibrated with 0.05% TFA. The column was washed with 0.05% TFA and absorbed materials were eluted stepwise with 20%, 40% and 60% acetonitrile containing 0.05% TFA. Each acetonitrile fraction showing peak at 280 nm was pooled and further subjected to Bruker Reflex IV matrix assisted laser desorption ionization (MALDI) mass spectrometer.

1.2.2 Reversed phase high performance liquid chromatography

High-performance liquid chromatography (HPLC) is a form of column chromatography used frequently in biochemistry and analytical chemistry. HPLC is used to separate components of a mixture by using a variety of chemical interactions between the substance being analyzed (analyte) and the chromatography column.

Reversed phase HPLC (RP- HPLC) consists of a non-polar stationary phase and a moderately polar mobile phase. Typical stationary phases are non-polar hydrocarbons (such as C_{18}, C_8, C_4, etc.). RP-HPLC operates on the

principle of hydrophobic interactions which result from repulsive forces between a relatively polar solvent, the relatively non-polar analyte, and the non-polar stationary phase. The retention time is therefore longer for molecules which are more non-polar in nature, allowing polar molecules to elute more readily.

Acetone-precipitated sample was dissolved in 1 M acetic acid. The acetic acid fraction was then applied to Lichrosorb C_{18} column, pre-equilibrated with 0.1% TFA. The column was washed with 0.05% TFA and absorbed materials were eluted linear gradient with 0-100% acetonitrile containing 0.05% TFA. Each acetonitrile fraction showing peak at 280 nm was pooled and further subjected to Bruker Reflex IV matrix assisted laser desorption ionization (MALDI) mass spectrometer. Both protein samples from androgenic tissues and vas deferens were compared to find the unique protein in androgenic gland.

1.2.3 Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE stands for sodium dodecyl sulfate polyacrylamide gel electrophoresis and is useful for molecular weight analysis of proteins. SDS is a detergent that dissociates and unfolds oligomeric proteins into its subunits. The SDS binds to the polypeptides to form complexes with fairly constant charge to mass ratios. The electrophoretic migration rate through a gel is therefore determined only by the size of the complexes. Molecular weights are determined by simultaneously running marker proteins of known molecular weights.

The acetone precipitated proteins from the androgenic tissues and vas deferens of both eyestalk-ablated and normal prawns were dialyzed against distilled water overnight to eliminate salts and impurities. To concentrate the proteins, the samples were speed-vac dried and resuspened in appropriate volume of distilled water.

Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (Schägger, 1987) is commonly used to separate proteins in the mass range 1–100 kDa. It is the preferred electrophoretic system for the resolution of proteins smaller than 30 kDa. Since the interested protein in this study is expected to be a peptide, the samples were then analyzed by Tricine–SDS-PAGE. The acetone extracted from each tissue was mixed with an equivalent NOVEX 2X Tricine-SDS sample buffer (see appendix) and heated for 5 min in a boiling water. Then, the samples were loaded on to 5% Tricine-SDS stacking gel and 15% separating gel (see appendix) and electrophoresed at constant current (35 mA) until dye reached bottom of the gel. The gel was stained in freshly prepared Coomassie Brilliant Blue staining solution for 1 h and then, destained in the destaining buffer for at least 2 h or until the background was clear.

1.3 Mass analysis by mass assisted laser desorption/ionization - time of flight (MALDI-TOF) techniques

Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) is a mass analysis technique that was pioneered by Hillenkamp *et al*. (1985). Basically, the analyte is mixed with matrix solution to assist ionization and then dried on a target plate. A laser beam is fired at the sample crystals simultaneously with the application of a high voltage pulse. The time it takes for sample ions to drift through the flight tube to the detector is proportional to their molecular weight.

Samples were dissolved in 0.1% trifluoroacetic acid (TFA), 0.5 µl of this solution were mixed with 0.5 µl of matrix solution (10mg/ml of sinapinic acid or 2, 5-dihydroxybenzoic acid in acetonitrile/0.1% TFA, 50:50). Finally, 1 µl of sample mixture was deposited on multi-well stainless steel MALDI plate and air dried. Measurements were performed on a time of flight Bruker Reflex (Bruker, Germany) in the linear mode with a mass range from 2000 to 12,000 Da, at 20 kV of acceleration potential.

1.4 Hormonal structure determination

1.4.1 Determination of disulfide bridges

The presence of disulfide bridges in the purified AGH was evaluated by following the change in molecular mass of the hormone after reduction of the disulfide bonds and *S*-carbamidomethylation.

The RP-HPLC purified 10.2 kDa protein from androgenic tissues was added with 10 µl of dithiothreitol (DTT) solution (1 mg/ml, 0.1 M Tris-HCl buffer, pH 9.0). The resulting mixture was incubated at 37 °C for 30 min. Next, 10 µl of sodium iodoacetate solution (2.5 mg/ml, 0.1 M Tris-HCl buffer, pH 9.0) was added to the reaction mixture, and incubation was continued at 37 °C for 30 min. The molecular mass of reduced protein was analyzed by matrix assisted laser desorption ionization (MALDI) mass spectrometer.

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1.4.2 Determination of glycan moiety containing in the hormone by enzymatic deglycosylation

To evaluate whether the purified AGH is a glycoprotein, the hormone was treated with a glycosidase enzyme and detected for the change in molecular mass of the hormone.

The RP-HPLC purified 10.2 kDa protein from androgenic tissues was dissolved in 20 µl of 20 mM ammonium bicarbonate, pH 8.0 containing 0.025% SDS and 100 mM 2-mercaptoethanol) before deglycosylated using 1 unit of N-glycosidase F (Sigma, Germany) for 24 h at 37 °C. The enzymatic deglycosylated protein was analyzed by MALDI-TOF MS.

1.4.3 Mass spectrometry

The reaction mixtures from enzymatic deglycosylation and reductive carboxymethylation were analyzed by matrix assisted laser desorption ionization (MALDI) mass spectrometer equipped with a delayed extraction source and 337 nm pulsed nitrogen laser. The instrument Bruker Reflex IV MALDI was run in the linear mode using 20 kV acceleration. The 1 µl of deglycosylated or reductive proteins were mixed with 1 µl of matrix solution (20 mg/ml of sinapinic acid in acetonitrile:water, 50:50, v/v). Finally, 0.5 µl of the mixture was deposited onto the MALDI target plate. All spectra are the results of signal averaging of 200 shots.

1.4.4 Identification of androgenic gland hormone by peptide mass fingerprinting using endoproteinase Lys-C digestion

Peptide Mass Fingerprinting (PMF) is a technique used to identify proteins by matching their constituent fragment masses (peptide masses) to the theoretical peptide masses generated from a protein or DNA database. The first step in PMF is that an intact, unknown protein is cleaved with a proteolytic enzyme to generate peptides. With PMF, heterogeneity is most commonly imparted to the unknown protein with a trypsin digestion. A PMF database search is usually employed following MALDI TOF mass analysis.The premise of peptide mass finger printing is that every unique protein will have a unique set of peptides and hence unique peptide masses. Identification is accomplished by matching the observed peptide masses to the theoretical masses derived from a sequence database.

The RP-HPLC purified 10.2 kDa protein from androgenic gland was dissolved in 30 µl of 0.1 M Tris-HCl buffer (pH 9.0) and 1 µg endoproteinase Lys-C (Sigma, Germany). The mixture was maintained at 37 °C for 18 h. After drying down, the samples were resuspended in 5 µl 0.1% TFA. The samples were mixed with 10 volumes of saturated 2, 5-Dihydroxybenzoic acid (DHBA) matrix solution and spotting 0.5 µl of the mixture onto the MALDI target plate. The MALDI-TOF MS was run in the positive refractor mode. The matching of the experimental peptide

mass with the *in silico*-derived tryptic peptide masses from the database was performed with MASCOT software (http://www.matrixscience.com). The NCBI non-redundant protein database was searched within a mass tolerance of 100 ppm; protein molecular weight search window of 12,000 Dalton; three missed cleavage was allowed. The oxidation of methionine was considered as modifications. Peptide masses were stated to be monoisotopic.

1.5 Identification of androgenic gland hormone by *de novo* sequencing

1.5.1 In-gel digestion

Protein bands from SDS-PAGE were cut into smaller pieces (1 mm^3 – 2 mm^3) and each was placed in a 1.5 ml microcentrifuge tube. Then, 200 µl of washing solution were added and the gel pieces were rinsed overnight at room temperature.The washing solution was removed and another 200 µl of washing solution were added and the gel pieces were further incubated for 2-3 h at room temperature. After the washing solution was removed, 200 µl of acetonitrile were added to dehydrate the gel pieces for 5 min and then, acetonitrile was removed. Thirty microlitres of 10 mM DTT were added to reduce the protein for 30 min and then, the solution was removed. Thirty microlitres of 100 mM iodoacetamide were added to alkylate the protein for 30 min and then, the solution was removed. Two hundred microlitres of acetonitrile were added to dehydrate the gel pieces for 5 min and then, acetonitrile was removed. To rehydrate the gel pieces, 200 µl of 100 mM ammonium bicarbonate were added and incubate for 10 min. After the solution was removed, 200 µl of acetonitrile were added to dehydrate the gel pieces for 5 min. After acetronitrile was removed, 30 µl of the trypsin solution kept on ice were added to the sample to rehydrate on ice for 20 min with occasional vortex mixing. Then, 5 µl of 50 mM ammonium bicarbonate were added to the sample for 20 min with occasional vortex mixing. The digestion was carried out overnight at 37°C. Sixty microlitres of the extraction buffer were added to the digest and incubated for 20 min with occasional gentle vortex mixing. The supernatant was collected and transferred into the new tube. Forty microlitres of the extraction buffer were added to the gel pieces again and

incubated for 20 min with occasional gentle vortex mixing. The supernatant was collected and transferred into the same tube. The volume of the extract was reduced to less than 20 µl by evaporating in a vacuum centrifuge. The protein-digested extract was stored in -80 ^{o}C.

1.5.2 *De novo* sequencing

Early *de novo* protein sequencing measurements relied on Edman degradation of the protein, but mass spectrometry (MS) has reduced the need for this technique because it is more sensitive and provides higher sample throughput. It can also cope better with protein mixtures and with modifications to the protein N-terminus. Straightforward MS measurement of the mass spectrum of the collection of proteolytic peptides resulting from enzymatic digestion of an unknown protein is often sufficient for protein identification. Along with a MALDI measurement of the overall protein mass, it is also the usual first step in *de novo* sequencing of the protein, yielding the masses of the individual peptides.

In-gel digestion of the expected protein purified from Tricine-SDS gels were analyzed on LC-MS-MS model LTQ (Finnigan) at BIOTEC, NSTDA.

2. Molecular cloning of androgenic gland hormone from cDNA

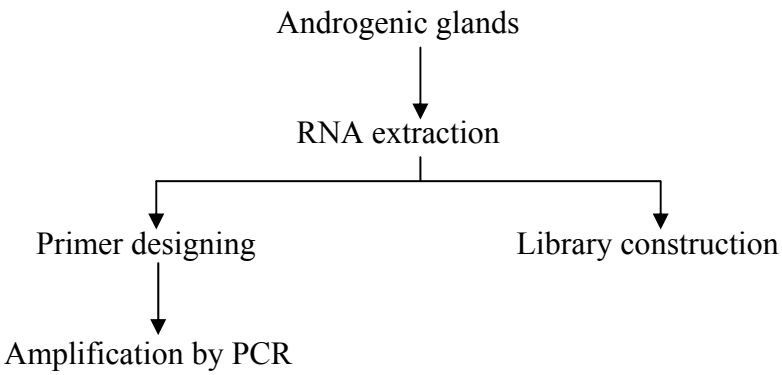


Figure 14 Flow chart of molecular cloning of androgenic gland hormone in *Macrobrachium rosenbergii*

2.1 RNA isolation

The integrity and purity of mRNA used as starting material is an important element of high-quality cDNA synthesis. Fresh deionized (e.g. MilliQ-grade) water or water treated with DEPC (diethyl pyrocarbonate) was used directly. All glassware were soaked with 0.5 M NaOH, followed by deionized water then baked at 160-180 °C for 4-9 h. Single-use pipette tips and microcentrifuge tubes were used. Gloves were worn to avoid contamination and degradation of RNA.

2.1.1 Isolation of total RNA from androgenic glands of giant freshwater prawn

Total RNA was extracted from androgenic glands of *M. rosenbergii* using TRIzol reagent. TRIzol reagent is a mono-phasic solution of phenol and guanidine isothiocyanate, is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (1987). During sample homogenization or lysis, TRIzol reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation, the solution was separated into an aqueous phase and an organic phase.

RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol. After removal of the aqueous phase, the DNA and proteins in the sample can be recovered by sequential precipitation.

Total RNA was extracted from androgenic glands from 12 male prawns which has average weight about 100 g per prawn using TRIzol reagent and was used as a template for RT-PCR. Total weight of extracted gland is 211 mg. Eighty nine microlitres of androgenic glands were homogenized with TRIzol reagent using pestle in 1.5-ml microcentrifuge tube and incubated for 5 min at room temperature to dissociate nucleoprotein complex. Then, the mixture was centrifuged at 12000 ×g for 15 min at 4 °C. The supernatant was added with 0.2 ml of chloroform (for 1 ml TRIzol reagent), shaked vigorously for 15 s and then incubated for 2-3 min at 15-30 °C. The mixture was centrifuged at 12000 ×g for 15 min at 4 °C. The upper aqueous phase was transferred to a new 1.5-ml microcentrifuge tube. The RNA was precipitated by mixing with 0.5 ml of isopropanol (for 1 ml TRIzol reagent). The sample was incubated at -20 °C for 1 h. Total RNA was precipitated by centrifugation at 12000 ×g at 4 °C for 10 min. After the supernatant was removed, the RNA pellet was washed twice with 75% (v/v) ethanol and centrifuged at 7500 ×g for 5 min at 4 °C. The washed RNA pellet was air-dried until ethanol evaporated completely and dissolved in nuclease-free water by incubating for 10 min at 55-60 °C. Total RNA from *M. rosenbergii* androgenic glands was further isolated for mRNA or transcribed to cDNA immediately.

2.1.2 Isolation of messenger RNA

Messenger RNA was isolated from the total RNA of *M. rosenbergii* androgenic glands using NucleoTrap mRNA Purification Kits (MACHEREY-NAGEL). Most of eukaryotic mRNA molecules contain poly(A) at their 3´ends. Thus most mRNAs can be isolated from a total RNA using their poly(A) tracks. Latex beads covalently modified with oligo(dT) residues in this kit will bind to poly(A) RNA under high-salt conditions. So, poly(A) RNA can be eluted with water

or low salt buffer. The quantity of poly(A) RNA is usually between 1-5% of total cellular RNA. The percentage of poly(A) RNA present depends on cell type, the growth or physiological state of the cell and storage conditions of the cell material.

According to the protocol recommended by the manufacturer, total RNA processed a 200-500 µl in nuclease-free water was added the same volume of RM0 binding buffer to adjust binding condition. The total RNA mixture was added to oligo(dT)-latex beads suspension (15 µl oligo(dT)-latex beads per 100 µg total RNA). After mixing, the mixture was heated at 68 °C for 5 min and incubated at room temperature for 10 min to let the oligo(dT)-latex beads bind to poly(A) RNA. After incubation, the tube was centrifuged for 15 s at 2,000 ×g, then for 2 min at 11,000 ×g. To wash the sample, supernatant was discarded and pellet was dissolved completely in 600 µl of washing buffer RM2. The oligo(dT) latex bead suspension mixture was transferred onto the NucleoTrap microfilter and centrifuged for 15 s at 2,000 ×g, then for 2 min at 11,000 ×g. After the flowthrough was discarded, 500 µl of washing buffer RM3 was added to the oligo(dT) latex beads and resuspended directly on the NucleoTrap microfilter by pipetting up and down until the solution became milky and no pellet was visible. Then, the tube was centrifuged for 15 s at 2,000 ×g, then for 2 min at 11,000 ×g. After the flowthrough was discard, 500 µl of washing buffer RM3 was added to the oligo(dT) latex beads and resuspended completely as described previously. The NucleoTrap microfilter was centrifuged for 15 s at 2,000 ×g and for 2 min at 11,000 ×g, then the flowthrough was discard. To completely remove the washing buffer, NucleoTrap microfilter was centrifuged for 1 min at 11,000 ×g. Before elution of pure poly(A) RNA, NucleoTrap microfilter was transferred to a clean RNase-free 1.5-ml microcentrifuge tube. The 20 µl of prewarmed (68 °C) RNase-free water per 10 µl of oligo(dT) latex beads was added and oligo(dT) latex beads was resuspended completely by pipetting up and down (elution buffer becomed milky). NucleoTrap microfilter column was incubated at 68 °C for 7 min and centrifuged for 1 min at 11,000 ×g before collecting eluate. The mRNA can be used immediately or stored at -70 ^{o}C.

2.2 Qualification and quantification of RNA

Two methods are widely used to measure the amount of nucleic acid concentration. If the sample is pure (i.e., without the contaminants such as proteins, phenol, or other nucleic acids), spectrophotometric measurement of the amount of (UV) irradiation absorbed by the bases is simple and accurate. If the amount of nucleic acid is very small or if the sample contains significant quantities of impurities, the amount of nucleic acid can be estimated from the intensity of fluorescence emitted by ethidium bromide (EtBr).

2.2.1 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to identify, separate, and purify RNA fragment. Agarose was mixed with 1X Tris-acetate EDTA (TAE) buffer (see Appendix) to an appropriate concentration for separation the particular size of RNA fragment and heat in a microwave until complete solubilization. While the agarose solution was cooling, a clean and dry gel-casting tray was sealed the ends with tape and an appropriate comb was selected for forming the sample slot in the gel. A position of the comb was 0.5-1.0 mm above the plate. The warm agarose (55 °C) was poured into the casting tray. After the gel set completely, the comb was removed carefully and the ends of the casting tray were unsealed. The gel is placed in an electrophoresis chamber containing TAE buffer.

The RNA sample was mixed with 6X gel-loading buffer (see Appendix) and the sample mixture was loaded slowly into the slots of the submerged gel. Standard RNA marker was loaded into the slots on both the right and left sides of the gel. The lid of the gel chamber was closed and the electrophoresis was carried out in 1X TAE running buffer. The RNA should migrate toward the positive anode at 100 volts and the bromophenol blue should migrate from the wells into the body of the gel within a few min. The gel was run until the bromophenol blue and xylene cyanol FF have migrated to an appropriate distance through the gel. After finishing, the gel was stained in 2.5 μg/ml ethidium bromide (EtBr) solution for 15 min and destained by

submerging in an excessive amount of distilled water for 15 min. The nucleic acid bands were visualized under UV transilluminator.

The advantage of using EtBr in gel is that RNA bands can be stained and monitored with a UV lamp. Because the amount of fluorescence is proportional to the total mass of RNA, the quantity of RNA can be estimated by comparing the fluorescent intensity of the RNA sample with a series of exact concentration of standard RNA. It is used in the detection of nanogram levels of RNA.

2.2.2 Spectrophotometric determination of RNA concentration

RNA concentration was estimated by UV spectrophotometer at the absorbance 260 nm (A260). An absorbance of 1.0 corresponded to 40 μg/ml of RNA (Sambrook and Russel, 2001). The RNA concentration of each sample was calculated in μg/ml by the following equations:

$$\text{RNA concentration} = A260 \times \text{dilution factor} \times 40\ \mu g/ml$$

The ratio of the A260 and A280 nm can be used as an indicator of nucleic acid purity. The ratio of pure nucleic acid should be between 1.8-2.0. A low A260/A280 ratio may indicate protein or phenol contamination. The measurements at A260 were quantitated for relatively pure nucleic acid preparation in microgram quantity RNA was stored at -20 °C. For long term storage, the total RNA was precipitated and kept in absolute ethanol at -70 °C.

2.3 Cloning and sequencing of the partial cDNA fragments encoding androgenic gland hormone

2.3.1 Oligonucleotide primers design

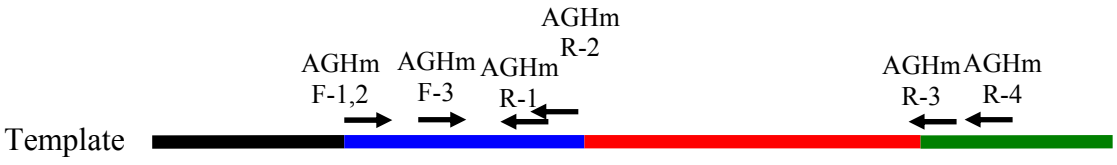
The oligonucleotide primers were designed based on the conserved sequences of AGHs from *Armadillidium vulgare*, *Porcellio scaber* and *P. dillatatus* (GenBank accession no. AB029615, AB089810 and AB089811, respectively). Moreover, the nucleotide and amino acid sequences of *Cherex quadricarinatus* insulin-like androgenic gland factor (GenBank accession no. DQ851163) were used as a template for primer designing as well. For positive control, the primers based on the actin gene of *M. rosenbergii* (GenBank accession no. AY651918) were designed. All primers were shown in Fig.15 and described in Table 2.

Table 2 The designed oligonucleotide primers for amplifying the androgenic gland hormone gene of *M. rosenbergii*.

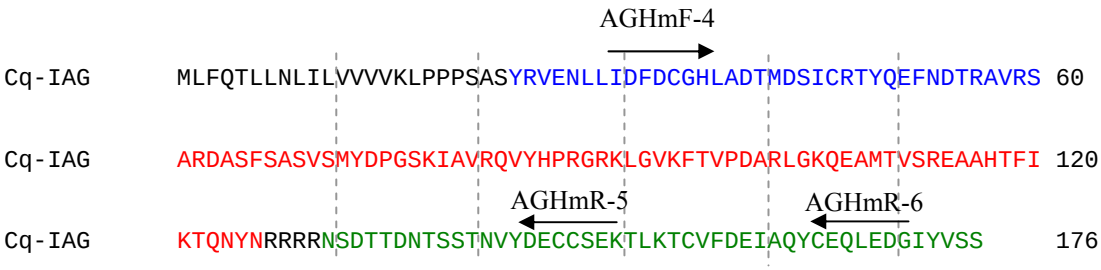
Primer Name	Sequence 5′ →3′	Nucleotide Position*	Tm (^{o}C)
Positive control			
Actin-F (forward)	CCCAGAGCAAGAGAGGTA	176-194	50
Actin-R (reverse)	GCGTATCCTTCGTAGATGGG	318-337	50
Based on A.vulgare, P. scaber and P. dillatatus			
AGHm-F1 (forward)	GCCTACCAGGTAATAGGTA	61-79	49
AGHm-F2 (forward)	GCCTACCAGGTA(ACT)(AGT) AGGTATG	61-81	52-56
AGHm-F3 (forward)	GTGCA(GT)TGTATATGCAACG AATT(AG)GG	121-144	55-58
AGHm-R1 (reverse)	CTTCGAAAGTCTCGACGTA	153-171	49
AGHm-R2 (reverse)	(TC)CTGTT(AT)GGCCA(AT)GG(AG) CAAGG(TC)TT	169-191	56-61
AGHm-R3 (reverse)	GT(AG) (AG)AA GGC (GT)AT (AGC) TC ACG TTT C	349-360	49-57
AGHm-R4 (reverse)	G(CT)TC(GT)GT(AG)C(GT)AAT(AG) TTGCAACA(TC)TC	364-388	51-61
Based on C. quadricarinatus			
AGHm-F4 (forward)	AT(ACT)GA(CT)TT(CT)GA(CT)TG(CT) GG(AGCT)CA	91-110	44-56
AGHm-F5 (forward)	CA(AG)GA(AG)TT(TC)AA(TC)GA(TC) AC(AGCT)(AC)G	148-167	40-58
AGHm-R5 (reverse)	TT(CT)TC(AGCT)GA(AG)CA(AG)CA(CT) TC(AG)TC	421-440	44-56
AGHm-R6 (reverse)	CC(AG)TC(CT)TC(AGCT)A(AG)(CT)TG (CT)TC(AG)CA	493-512	46-60

* Calculated position starts at start codon

(A)



(B)



(C)

(D)

Figure 15 The positions of primers on (A,B) Amino acid sequence alignment of *A. vulgare*, *P. scaber* and *P. dillatatus* androgenic gland hormones (Arv-AGH, Pos-AGH and Pod-AGH, respectively) and (C,D) amino acid sequence of *Cherex quadricarinatus* insulin-like androgenic gland factor.

——→ : indicates forward primer

←—— : indicates reverse primer

Moreover, the internal peptide sequences derived from trypsin digestion were used to design some degenerated primers specific to the AGH as shown in Table 3.

Table 3 Sequences of the degenerated primers designed from the peptide fragment sequences derived from protein of *Machrobrachium rosenbergii* androgenic tissue

Protein	Name	Nucleotide sequence of the degenerated primer (5′----3′)
10D	Forward	
	10D_F1	TN TTY GAY GAR ACN AC
	10D_F2	GTN TGG GAY TTY MG
	10D_F3	TN GAR TGG GAR WSN MG
	10D_F4	GAR TAY YTN TAY CCN MG
	Reverse	
	10D_R1	GTN GTY TCR TCR AAN A
	10D_R2	CKR AAR TCC CAN AC
	10D_R3	CKN SWY TCC CAY TCN A
	10D_R4	CKN GGR TAN ARR TAY TC
15D	Forward	
	15D2_F1	GTN YTN GAY TGG CAR CC
	15D2_F2	GCN GGN WSN GGN AA
	15D2_F3	GGN GCN GTN YTN CAN GG
	Reverse	
	15D2_R1	GGY TGC CAR TCN ARN AC
	15D2_R2	TTN CCN SWN CCN GC
	15D2_R3	CCN TGN ARN ACN GCN CC

Table 3 (Continued)

Protein	Name	Nucleotide sequence of the degenerated primer (5′----3′)
AG9.7	Forward	
	AG9.77_F1	AAY YTN CAR CAR CAR MG
	Reverse	
	AG9.77_R1	CKY TGY TGY TGN ARR TT
AG10.7	Forward	
	AG10.72_F1	AAR CAR TTY GAY GCN GC
	Reverse	
	AG10.72_R1	GCN GCR TCR AAY TGY TT
AG12.5	Forward	
	AG12.5_F1	GAY GAR GAY YTN GC
	Reverse	
	AG12.5_R1	GCN ARR TCY TCR TC
AG13.6	Forward	
	AG13.8_F1	ACN CCN GAY AAR AC
	AG13.8_F2	TN GAR CAR GAR AA
	AG13.8_F3	TN GGN GGN TAY CCN AC
	Reverse	
	AG13.8_R1	GTY TTR TCN GGN GT
	AG13.8_R2	TTY TCY TGY TCN A
	AG13.8_R3	GTN GGR TAN CCN CCN A

2.3.2 PCR amplification

The partial genomic DNA sequence of AGH gene of *M. rosenbergii* were amplified by polymerase chain reaction (PCR), using cDNA of *M. rosenbergii*, as a template. The primers were paired for amplifying the AGH gene of *M. rosenbergii*. The amplified reaction of 10 µl contained 0.1 µM of dNTP (dATP, dCTP, dGTP, dTTP), 2 µM of primers and 0.25 U of RealTaq DNA polymerase. The reaction mixture was amplified in a Perkin Elmer Thermocycle 480. PCR procedure was as followed: preheated at 94 ^{o}C for 7 min, followed by 30 cycles of denaturing at 94 ^{o}C for 30 s, annealing at annealing temperature (Ta) for 30 s, and extension at 72 ^{o}C for 1 min. The final extension was carried out at 72 ^{o}C for 7 min. The PCR product was separated on 2% agarose gel and then the DNA fragment was purified from agarose gel using NucleoSpin Extraction Kit (MACHEREY - NAGEL, Germany).

2.3.3 Purification of DNA fragment from agarose gel

Purification of DNA fragment from agarose gel was performed according to NucleoSpin Extraction Kit instruction. In brief, the desired DNA fragments on agarose gel (from section 2.5.2) were excised with razor blade and the agarose gel containing the band was transferred to a microcentrifuge tube. Three hundred microliters of NT1 buffer was added to every 100 µl of the agarose gel. The sample was incubated at 50 ^{o}C for 10 min or until the agarose gel was completely dissolved. The sample was loaded into NucleoSpin Extract Spin Column which was in a 2-ml collection tube. The column was centrifuged at 8,000 x g for 1 min at room temperature. After discarding the flowthrough, 600 µl of NT3 buffer were added to the column. The column was centrifuged at 11,000 x g for 1 min. After discarding the flowthrough, 200 µl of NT3 buffer were added to the column. The column was centrifuged at 11,000 x g for 2 min. The NucleoSpin Extract column was transferred to a clean 1.5-ml microcentrifuge tube. Thirty microlitres of nuclease-free water was added and the column was incubated for 1 min. The column was centrifuged at 11,000 x g for 1 min. DNA yields were determined by UV spectrophotometry and by agarose gel electrophoresis.

2.3.4 DNA ligation

The purified DNA fragment was ligated into pGEM-T easy vector (Promega) which possessed a single 3′ deoxythimidine (T) overhanged at both ends. This vector allowed easy cloning of PCR product based on the fact that *Taq* polymerase used in PCR tend to add an additional nucleotide, usually a deoxyadenosine (A) to the 3′- end of each strand that it synthesized. Therefore, a double-stranded PCR product has a single adenosine nucleotide overhang. The molar ratio of the insert DNA to the vectors is usually 1:3 to 3:1. The amount of inserted DNA depended on the length of the inserted DNA fragment. The quantity of inserted DNA was estimated in ng by the following equation:

$$\text{x ng of insert DNA} = \frac{(25 \text{ ng of vector})(\text{y bp of insert DNA}) \times \text{insert:vector molar ratio}}{3000 \text{ bp of pGEM-T vector}}$$

Where x was the amount of insert DNA of y bp to be ligated for 1:3 (vector:insert molar ratio).

The 5 µl of each ligation reaction composed of inserted DNA, 2.5 µl of 2X T4 DNA ligase buffer,0.5 µl of pGEM-T vector (25 ng), and 0.5 µl of T4 DNA ligase (3 U/µl). Sterile water was added to the final volume of 5 µl. Each mixture was gently mixed by pipetting and then incubated at 16 ^{o}C, overnight or at 25 ^{o}C for 3 h.

2.3.5 Bacterial transformation

The tube containing the ligation reaction (from section 2.6.4) was centrifuged to collect contents at the bottom of the tube. *Escherichia coli* HIT JM109 (UBI, Canada) was used as host cell. Competent cell vial with was thawed at room- temperature tap water or water bath for 10~20 seconds until 1/3 thawed. Fifty microlitres of the cell suspension was mixed with 1.5 µl of ligation mixture. The mixture was mixed and placed on ice for 10 minutes. Then, the transformed mixture

was plated on LB ampicillin agar plates (see Appendix) spreaded with 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-Gal. The plates were incubated at 37 °C for 8-24 h. Recombinant clones were analyzed by blue/white screening. The transformant colonies were counted and selected randomly to check the transformation of the inserted DNA by colony PCR analysis.

2.3.6 Determination of inserted DNA size by PCR

To determine the size of the inserted cDNA in plasmid pGEM-T, colony PCR was performed directly using the bacterial cell from colonies as template by amplification with flanking primers situation on polylinker of the vector, SP6 (5′>CGATTTAGGTGACACTATAG <3′) and T7 (5′>TAATACGACTCACTATAG GG<3′). The reaction mixture was conducted in 5 µl final volume. The final concentration of each component was 1X Real Taq PCR buffer with $MgCl_2$, 0.1 µM dNTP mix, 0.2 µM each SP6 and T7 primers, 1.25 U Real Taq DNA polymerase (RBC, Taiwan). PCR reaction was as followed: preheated at 94°C for 3 min, followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 50 °C for 30 s, and extension at 72°C for 1 min, followed by final extension at 72 °C for 7 min. The PCR products were separated on a 2% agarose gel in 1X TAE buffer and stained in 0.5 µg/ml EtBr solution as described in section 2.4.

2.4 DNA Sequencing

2.4.1 Plasmid DNA preparation

The recombinant clones containing expected size product from section 2.6 were propagated and purified using plasmid DNA extraction Kit (MACHEREY-NAGEL, Germany). Each clone, freshly cultured overnight in LB broth containing 100 µg/ml ampicillin (1.5 ml), was transferred to a microcentrifuge tube and centrifuged at 11000 ×g for 1 min. The bacterial cell pellet was resuspended by vigorous vortex in 250 µl of bufferA1 and then 250 µl of buffer A2 was added and mixed gently by inverting the tube 6-8 times. The sample was incubated at room

temperature for 5 min. After incubation, 300 µl of buffer A3 was added and mixed gently by inverting the tube 6-8 times. The supernatant was collected by centrifugation at room temperature for 10 min. The supernatant was transferred into Nucleospin plasmid column which was placed in a 2 ml collected tube and centrifuged at 11000 ×g for 1 min. The column was washed with 500 µl prewarm buffer AW and centrifuged at 11000 ×g for 1 min. The flow-through was discarded and the 600 µl of buffer A4 was added to the column and centrifuged at 11000 ×g for 1 min. Residual washed buffer was removed from the column by additional centrifuged at 11000 ×g for 2 min and then the column was placed into a new 1.5 ml microcentrifuge tube. The plasmid DNA was eluted by adding 50 µl of AE buffer (10 mM Tris-HCl, pH 8.5) and incubated at room temperature for 1 min. After incubation, the tube was centrifuged at 11000 ×g for 1 min and the plasmid DNA was collected.

2.4.2 Sequencing of DNA

The sequence electrophoresis and analysis were performed at the Bioservice unit (BSU) by an ABI™PrismTM 377 Genetic Analyzer and Macrogen DNA sequencing service (Korea) by 6 Applied Biosystems 3730 and 9 ABI 3700. The data were recorded by the computer and the results were displayed either as a graph or as a text sequence.

2.4.3 DNA Sequence Analysis

Homologous sequences were sought using the Basic Alignment Search Tool (BLAST) (Altschul et al., 1997). Related AGH sequences were cited from the GenBank database. A multiple sequence alignment was made with CLUSTAL W (Thompson et al, 1994). Derived protein sequences were analyzed by the BLAST algorithm (Altschul et al., 1997) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

2.5 cDNA library construction

The rapid identification of genes expressed in a variety of organisms has been achieved by several laboratories during the past few years by the systematic sequencing of cDNA libraries. The general strategy of such approaches is based on the identification by a "single pass" sequencing of random cDNA clones that results in the production of short partial sequences identifying a specific transcript, generally known as Expressed Sequence Tag (EST). Since cDNA libraries can be prepared from different tissues or developmental stages of a single organism, this approach can be useful for the construction of catalogues of tissue-specific or stage-specific genes.

cDNA library of *M. rosenbergii* androgenic glands was constructed by the SMART cDNA library construction kit (Clontech). This kit provides a method for producing high quality, full-length cDNA with complete 5' ends cDNA libraries from nanograms of total or poly(A) RNA.

The overview of full-length cDNA library construction was shown in Fig.16.

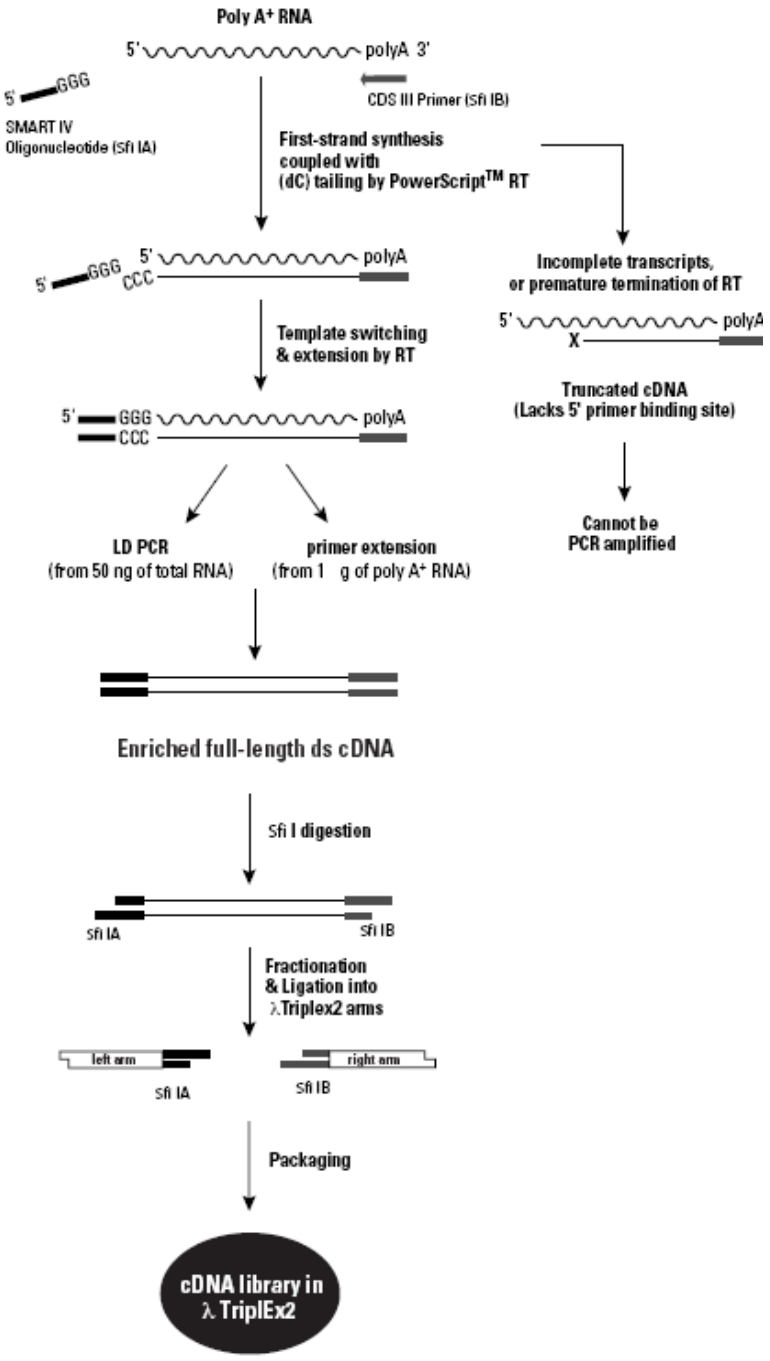


Figure16 Flow chart of the SMART cDNA library construction kit protocols.

2.5.1 First-Strand cDNA Synthesis

First-strand cDNA was reverse transcribed from mRNA following the manufacturer's protocol. Fifty nanograms of mRNA (section 2.1.2) was combined with 1 µl of SMART IV oligonucleotide and 1 µl of CDS III/3' PCR Primer. Steriled water was added to final volume of 5 µl and mixed briefly. The reaction mixture was incubated at 72 °C for 2 min and then cooled on ice for 2 min. After incubation, the tube was centrifuged briefly to collect the contents at the bottom. Then, 2.0 µl of 5X first-strand buffer, 1.0 µl of 20 mM DTT, 1.0 µl of 10 mM dNTP mix and 1.0 µl of PowerScript reverse transcriptase was added to the RNA-primer mixture. The reaction mixture was gently mixed by up-down pipetting and incubated at 42 °C for 1 h in an air incubator. After incubation, the tube was placed on ice to terminate first-strand synthesis.

2.5.2 Second-Strand cDNA Synthesis Long-Distance PCR

This protocol is appropriate for limiting the amount of RNA starting material. The second-strand cDNA was synthesized using the 2 µl first-strand reaction (from section 2.6.1). Then, combined with 80 µl of deionized water, 10 µl of 10X Advantage 2 PCR buffer, 2 µl of 50X dNTP mix, 2 µl of 5' PCR primer, 2 µl of CDS III/3' PCR primer and 2 µl of 50X Advantage 2 polymerase mix and mixed briefly. The reaction mixture was centrifuged briefly to collect the contents at the bottom of the tube. The tube was placed in a preheated (95 °C) thermal cycler for 20 s and followed by twenty cycles of denaturing at 95 ^{o}C for 15 s, annealing/extension at 68 ^{o}C for 6 min.

2.5.3 Proteinase K digestion

Two microlitres of 20 µg/µl proteinase K was added to 50 µl of amplified ds cDNA (2–3 µg) from section 2.6.2. The contents was mixed and incubated at 45 °C for 20 min. Then, 50 µl of deionized water was added to the tube. One hundred microlitres of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) was

added. The tube was mixed by continuous gentle inversion for 1–2 min and centrifuged at 14,000 rpm for 5 min at 4 ^{o}C to separate phases. The upper aqueous phase was carefully transferred to a new 0.5-ml microcentrifuge tube. One hundred microlitres of chloroform:isoamyl alcohol (24:1, v/v) was added and mixed thoroughly. The microcentrifuge tube was centrifuged at 14,000 rpm for 5 min and upper phase was transferred to a new microcentrifuge tube. Ten microlitres of 3 M sodium acetate, 1.3 µl of 20 µg/µl glycogen, and 260 µl of 95% ethanol was added and centrifuged at 14,000 rpm for 20 min at room temperature. The supernatant was removed carefully and the pellet was gently washed with 100 µl of 80% (v/v) ethanol, air-dried and then dissolved in 79 µl of water and stored at –20 ^{o}C.

2.5.4 *Sfi* I digestion

The following components were combined in a fresh 0.5-ml tube: 79 µl of cDNA (Section 2.6.3), 10 µl of 10X *Sfi* buffer, 10 µl of *Sfi* I enzyme and 1 µl of 100X BSA. After mixing, the tube was incubated at 50 °C for 2 h and then 2 µl of 1% xylene cyanol dye was added. The mixture was used immediately in the next step.

2.5.5 cDNA size fractionation by CHROMA SPIN-400

The CHROMA SPIN-400 column was prepared following the manufacture's procedure to adjust the appropriate condition (1 drop/40–60 s flow rate). One hundred microlitres of Sfi I-digested cDNA and xylene cyanol dye (Section 2.6.4) was applied to the column carefully after the storage buffer stopped dripping out. Then, the sample was allowed to be fully absorbed into the surface of the matrix. The tube was washed with 100 µl of column buffer and allowed the buffer to drain out of the column until there was no liquid left. The rack containing 16 collection tubes was placed under the column. Six hundred microlitres of column buffer was added and single-drop fraction was immediately collected in tubes number 1–16. The profile of the fractions was checked before proceeding with the experiment. On a 1.1% agarose/EtBr gel, 3 µl of each fraction was electrophoresed separately in adjacent wells, alongside 0.1 µg of a 1-kb DNA size marker. The gel was run at 150 V for 10

min. (Running the gel longer make it difficult to see the cDNA bands). The peak fractions were determined by visualizing the intensity of the bands under UV. The first four fractions containing cDNA was collected and pooled in a clean 1.5-ml tube. One tenth volume of 3 M sodium scetate pH 4.8, 1.3 µl of 20 mg/ml glycogen and 2.5 volume of 95% ethanol (–20°C) was added and mixed by gently rocking the tube back and forth. The tube was placed in –20 °C for 1 h and centrifuged at 14,000 rpm for 20 min at room temperature. The supernatant was discarded with a pipette and centrifuged briefly. All liquid was carefully removed and the pellet was allowed to air dry for 10 min. The pellet was resuspended in 7 µl of deionized water and mixed gently. The Sfi I-digested cDNA was ready to be ligated to the Sfi I-digested, dephosphorylated λTriplEx2 vector or stored at –20°C until the ligation step.

2.5.6 Ligation of cDNA to λTriplEx2 Vector

The ratio of cDNA to vector in the ligation reaction was a critical factor in determining transformation efficiency and the number of independent clones in the library. Three parallel ligations were set up using three different ratios of cDNA to vector (Table 4) to determine the optimal ratio of cDNA to vector in ligation reactions.A test ligation was set up to determine the efficiency of ligating the vector to the control insert. One microlitre of vector, 1 µl of control insert, 1.5 µl of deionized water, and other reagents listed in table 4 were mixed. All ligation reactions were incubated at 16 °C overnight. Each of the ligations was performed a separate λ-phage packaging reaction and titered the resulting phage.

Table 4 Ligations using three different ratios of cDNA to phage vector

Component	1st ligation (µl)	2nd ligation (µl)	3rd ligation (µl)
cDNA	0.5	1.0	1.5
Vector (500 ng/µl)	1.0	1.0	1.0
10X ligaton buffer	0.5	0.5	0.5
ATP (10 mM)	0.5	0.5	0.5
T4 DNA ligase	0.5	0.5	0.5
Deionized water	2.0	1.5	1.0
Total volume	5.0	5.0	5.0

2.5.7 Lamda(λ)-phage packaging reaction

The construction of highly representative gene libraries is essential to the successful isolation of cDNA or genomic clones from complex organisms. Bacteriophage lambda is an important cloning vehicle for this purpose, primarily because of the high efficiency with which cells can be infected by phage particles packaged in vitro. In vitro packaging refers to the use of a phage-infected E. coli cell extract to supply the mixture of proteins and precursors required for encapsidating lambda DNA.

In this study, the packaging reaction was performed by using Packagene Lamda DNA packaging system (Promega, USA) 1. Fifty microlitres Packagene® Extract was thawed on ice. Ligated DNA from section 2.6.6 was added to the extract for 0.5 ul and mixed by gently tapping the bottom of the tube several times. The extract/DNA mixes was incubated at 22°C (room temperature) for 3 hours. To each packaging mix, 445µl of phage buffer and 25µl of chloroform were added. DNA concentration was 1 µg/ml. The packaged phage was added by DMSO (7% v/v final concentration). The packaged phage was stored in this solution at -70°C until use or for up to 1 year.

2.5.8 Titering the Unamplified Library

The XL1-Blue host cells were prepared for trunsduction. A single colony from the working stock plate of XL1-Blue host cell was isolated and inoculated 15 ml of LB/$MgSO_4$/maltose broth in a 50-ml test tube or Erlenmeyer flask. Then, the cell broth was incubated at 37 °C overnight while shaking (at 140 rpm) until the OD_{600} of the culture reached 2.0. The cell-cultured broth was centrifuged at 5,000 rpm for 5 min. The supernatant was poured off and the pellet was resuspended in 7.5 ml of 10 mM $MgSO_4$.

The appropriate dilutions of each of the packaging extracts (from Section 2.6.7) were made in 1X lambda dilution buffer. In this study, an appropriate dilution for an unamplified λ lysate was 1:5, 1:10 and 1:20. One microlitre of the diluted phage was added to 200 µl of the XL1-Blue overnight culture, and the phage was allowed to adsorb at 37 °C for 10–15 min. Two millilitres of melted LB/$MgSO_4$ top agar were added, mixed by quickly inverting and immediately poured onto 90-mm LB/$MgSO_4$ plates prewarmed to 37 °C. The plates were quickly swirled after pouring to allow even distribution of the top agar. After that, the plates were cooled at room temperature for 10 min to allow the top agar to harden. The plates were inverted and incubated them at 37 °C for 6–18 hr. The plaques were counted and the titer of the phage (pfu/ml) was calculated

$$\text{pfu/ml} = \frac{\text{number of plaques x dilution factor x } 10^3 \text{ ml/ml}}{\text{ml of diluted phage plated}}$$

The titers should be less than 1–2 x 10^6 plaques (clones) altogether.

2.5.9 Conversion

Converting a λTriplEx/λTriplEx2 clone to a pTriplEx/pTriplEx2 clone requires excising and circularizing a complete plasmid from the recombinant phage. The plasmid is released by Cre recombinase-mediated recombination at *loxP*

sites. Release occurs automatically when recombinant phage is transduced into a bacterial host expressing Cre recombinase. In this system, *E. coli* BM25.8 growing at 31°C provides Cre recombinase activity. Conversion may be performed on either individual positive plaques picked from the secondary or tertiary screening plates, or the entire library. Released plasmids differ from pTriplEx by a 100-bp *loxP* insert at the Cla I site. Excised plasmids are propagated stably in recombinase-deficient *E. coli*.

The BM25.8 cells were prepared for conversion. A single colony from the working stock plate of BM25.8 cells was isolated and inoculated 10 ml of LB broth in Erlenmeyer flask. Then, the cell broth was incubated at 31 °C overnight while shaking (at 190 rpm). One microlitre of overnight culture was removed and used to inoculate 10 ml of LB medium (1:10 dilution) and continued growing at 31°C with shaking until the OD_{600} reached 1.2 (2–2.5 h). Transfect cells on the same day.until the OD_{600} of the culture reached 2.0. One hundred microliters of 1 M $MgCl_2$ were added to the 10 ml freshly grown BM25.8 culture (10 mM final concentration). One hundred microliters of BM25.8 cells were mixed with λTriplEx cDNA containing 2 x 10^6 pfu in a sterile 5-ml tube by pipetting. This mixture of cells and phage was incubated for 1 h at 31 ^oC without shaking. After incubation was complete, 500 µl of LB medium were added and incubated for 1 h at 31 ^oC with shaking at 190 rpm. At this point, conversion of the entire library to plasmid form was complete. Ten and one hundred microliters of converted cDNA library (diluted 1:100 in LB medium) were spreaded on 150-mm LB agar plates containing 50 µg/ml ampicillin. The LB agar plates were incubated overnight at 37 ^oC. The colonies were counted and determined the total number of converted recombinant clones per µl. Twenty to thirty thousand converted clones were plated on each 150-mm LB plate containing 50 µg/ml ampicillin (3–5 x 10^6 clones) and grown overnight at 37 ^oC (not longer than 14 h).

2.5.10 Determination of inserted DNA size by PCR

To determine the size of the inserted cDNA in pTriplEX plasmid, PCR was performed directly using the randomly picked clones as template by amplification with flanking primers situation on polylinker of the vector, SP6 (5′>CGATTTAGGTGACACTATAG <3′) and T7 (5′>TAATACGACTCACTATAG GG<3′). The reaction mixture was conducted in 5 µl final volume. The final concentration of each component was 1X Real Taq PCR buffer with $MgCl_2$, 0.1 µM dNTP mix, 0.2 µM each SP6 and T7 primers, 1.25 U Real Taq DNA polymerase (RBC, Taiwan). PCR reaction was as followed: preheated at 94 °C for 3 min, followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min, followed by final extension at 72 °C for 7 min. The PCR products were separated on a 1.2% agarose gel in 1X TAE buffer and stained in 0.5 µg/ml EtBr solution as described in section 2.4. The sequence analysis was performed at the Bioservice unit (BSU) by an ABITM PrismTM 377 Genetic Analyzer and Macrogen DNA sequencing service (Korea) by 6 Applied Biosystems 3730xl and 9 ABI 3700. The data were recorded by the computer and the results were displayed either as a graph or as a text sequence.

2.5.11 Sequencing of DNA

One thousand recombinant clones containing expected size product from section 2.6 were propagated and purified using plasmid DNA extraction Kit as mentioned above. Sequencing from both 3′ and 5′ end was applied to 960 independent colonies by Dr. Kazuei Mita, Insect Genome Research Unit, National Institute of Agrobiological Science , Tsukuba, Ibaraki JAPAN. However, the sequencing by 3' primer was not good due to long poly A-tail. Therefore, one-pass sequences were from only 5′ end. An average length of nucleotide sequences of the 776 readings was about 500 bp with poly A tail. After removal of vector-based sequences, 766 readings (98.7%) were analyzed further.

2.5.12 DNA Sequence Analysis

Homologous sequences were sought using the Basic Alignment Search Tool (BLAST) (Altschul *et al*., 1997). For identification of *M. rosenbergii* cDNA clones, the cutoff was set at an expected value ($E \leq 10^{-4}$). Identified clones generally shared a sequence identity >50% over a relatively long range >150 bp with the most similar sequence for BLASTX. ESTs that matched known or predicted genes were categorized into different groups according to functions. The nucleotide sequences of no significant similarity were translated and calculated the theoretical molecular weight using the ExPASy Proteomics Server (Gasteiger *et al*., 2005) and the most likely frame was selected. The signal peptide was theoretically predicted by SignalP 3.0 Server (Bendtsen *et al*., 2004). TargetP 1.1 (Emanuelsson *et al*., 2000) was used to predict the subcellular location of eukaryotic proteins. The location assignment is based on the predicted presence of any of the N-terminal presequences. The NetNglyc1.0 server was used to predict N-Glycosylation sites in proteins using artificial networks that examine the sequence context of Asn-X-Ser/Thr sequons. The deduced amino acid sequence was further considered by SMART (http://smart.embl-heidelberg.de/smart) (Schultz *et al.*, 2000). The deduced amino acid sequences of unknown sequence were compared with the pro-*Cq-IAG*, three pro-AGHs known in isopods (Manor *et al*., 2007.,Ohira *et al*., 2003; Okuno *et al*., 1997), internal amino acid sequences and cDNA amplified using primer based on *A. vulgare*, *P. scaber*, *P. dillatatus* and of *C. quadricarinatus* insulin-like androgenic gland factor. Moreover, the multiple sequence alignments were performed by ClustalW (Thompson *et al*., 1997).

The flow chart of the EST analysis was shown in Fig.17.

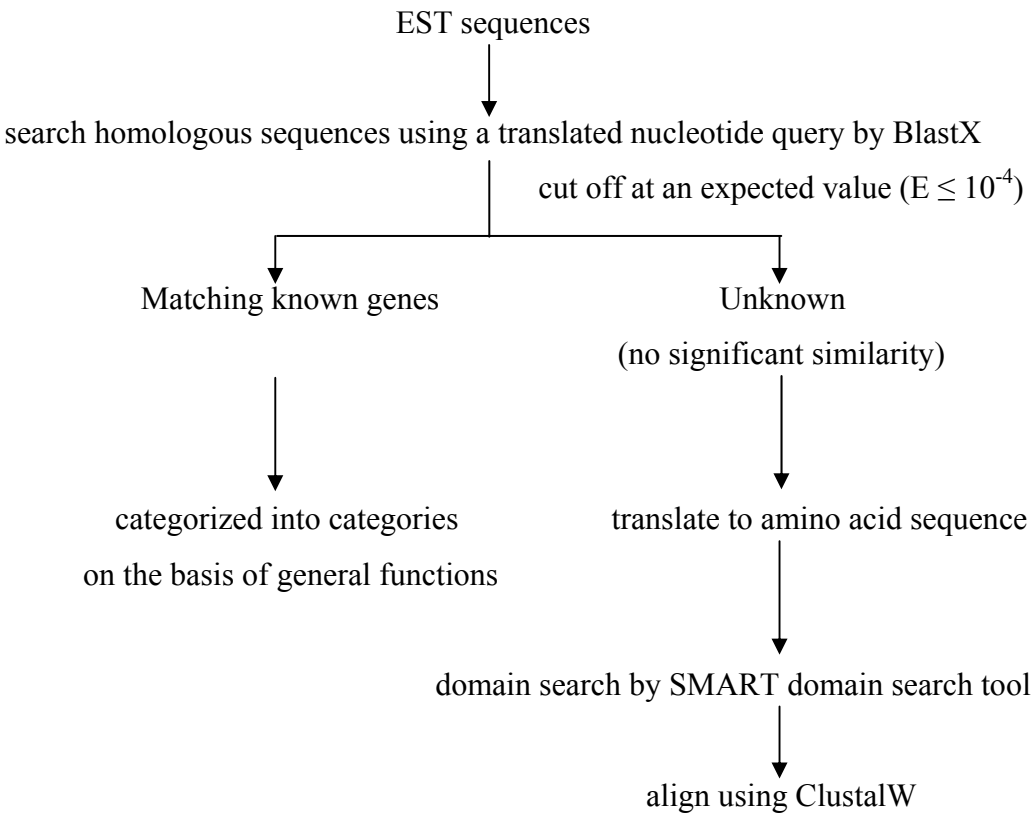


Figure 17 Flow chart of the *Macrobrachium rosenbergii* androgenic gland EST analysis

3. Androgenic gland hormone bioactivity determination

3.1 Sample preparation

Crude sample and pooled protein fractions were freeze-dried to eliminate acetronitrile and concentrate the samples. All samples were dissolved in sterile water to reach 1 mg/ml concentration.

3.2 Bioassay for androgenic gland hormone activity

The acid-alcohol, acetone extract of androgenic tissues and Sep-Pak C18 purified proteins were diluted in water and adjusted to pH 7. Two microliters of each sample was injected into 20-30 days old *M. rosenbergii* females at the position of the first ventrally abdominal segments by microsyringe. Before injection, young female prawns were shocked by dipping into ice-cold water. Eight injections were given, each at 10 day intervals, with the prawns being sacrificed 10 days after the final injection. After 80 days of injections, the animals were observed for indications of masculinization or inhibition of feminine trait.

RESULTS AND DISSCUSSION

Results

1. Extraction and purification of androgenic gland hormone from freshwater prawn

1.1 Extraction of androgenic gland hormone from freshwater prawn

In order to obtain larger amount of AGH (AGH), hypertrophy of androgenic glands of male freshwater prawn was induced by ablation of one eyestalk. The after 20 days, the gland and vas deferens each was dissected out.
Based on the assumption that AGH was specifically localized in the gland, it must be found only in the gland and not be found in the vas deferens. So, in this experiment both androgenic tissues (androgenic gland plus vas deferens) and vas deferens were extracted and difference in their protein patterns were compared.

The tissues of hypertrophied androgenic tissues and vas deferens were homogenized in acid-alcohol to prevent protease activity and remove large protein molecules. The supernatant was treated twice with cold acetone to precipitate the small proteins or peptides. After solubilization of the precipitate with acetic acid, the extract of each tissue was subjected to reverse phase column chromatography on Sep-Pak C18 cartridge. Protein contents of acetone androgenic tissues and vas deferens extracts were shown in Table 5. Total protein per mg of tissue of the acetone extract of androgenic tissues and vas deferens were nearly the same, 31 and 38 μg per mg tissue, respectively.

Table 5 Protein contents of the acetone androgenic tissues and vas deferens extracts of *Macrobrachium rosenbergii.*

	Androgenic tissues	Vas deferens
Number of prawns	150	150
Total tissue weight (mg)	7.4	13.9
Total protein content (mg)	231.1	531.0
Total protein (μg) per mg tissue	31	38

1.2 Purification of AGH by reverse phase chromatography

An aliquot (25 μg protein) of acetone androgenic tissues and vas deferens extracts each was analyzed by reverse phase HPLC on Sep-Pak C18 cartridge. Protein profile of stepwise eluted by 20%, 40% and 60% acetonitrile containing 0.05% TFA was shown in Fig. 18. The chromatograms obtained from the acetone androgenic tissues (Fig. 18A) and vas deferens extract (Fig. 18B) displayed peaks which were not well resolved. However, fractions of each peak were pooled and analyzed by MALDI-TOF MS. The MALDI-TOF MS spectrum of the acetone extracts, pooled unbound fractions and the acetronitrile fractions of the acetone androgenic tissues and vas deferens extracts were shown in Fig.19-23, respectively.

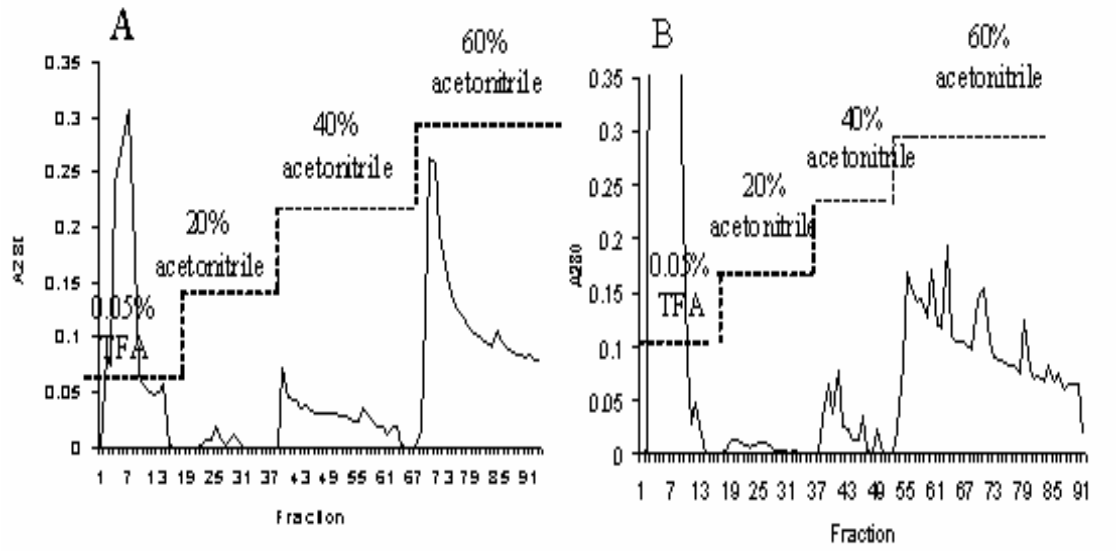


Figure 18 Reverse phase HPLC of acetone extracts of the androgenic tissues and vas deferens on Sep-Pak C_{18} catridge.

The acetone androgenic tissues (A) and vas deferens (B) extracts of the freshwater prawn (25 µg / 5 µl) were applied to a Sep-Pak C_{18} cartridge, pre-equilibrated with 0.05% TFA. The catridge was washed with the equilibration solvent and the adsorbed proteins were eluted stepwise with isocratic 20%, 40% and 60% acetonitrile containing 0.05% TFA at a flow rate of 1 mg/ml. Fractions of 2 ml were collected.

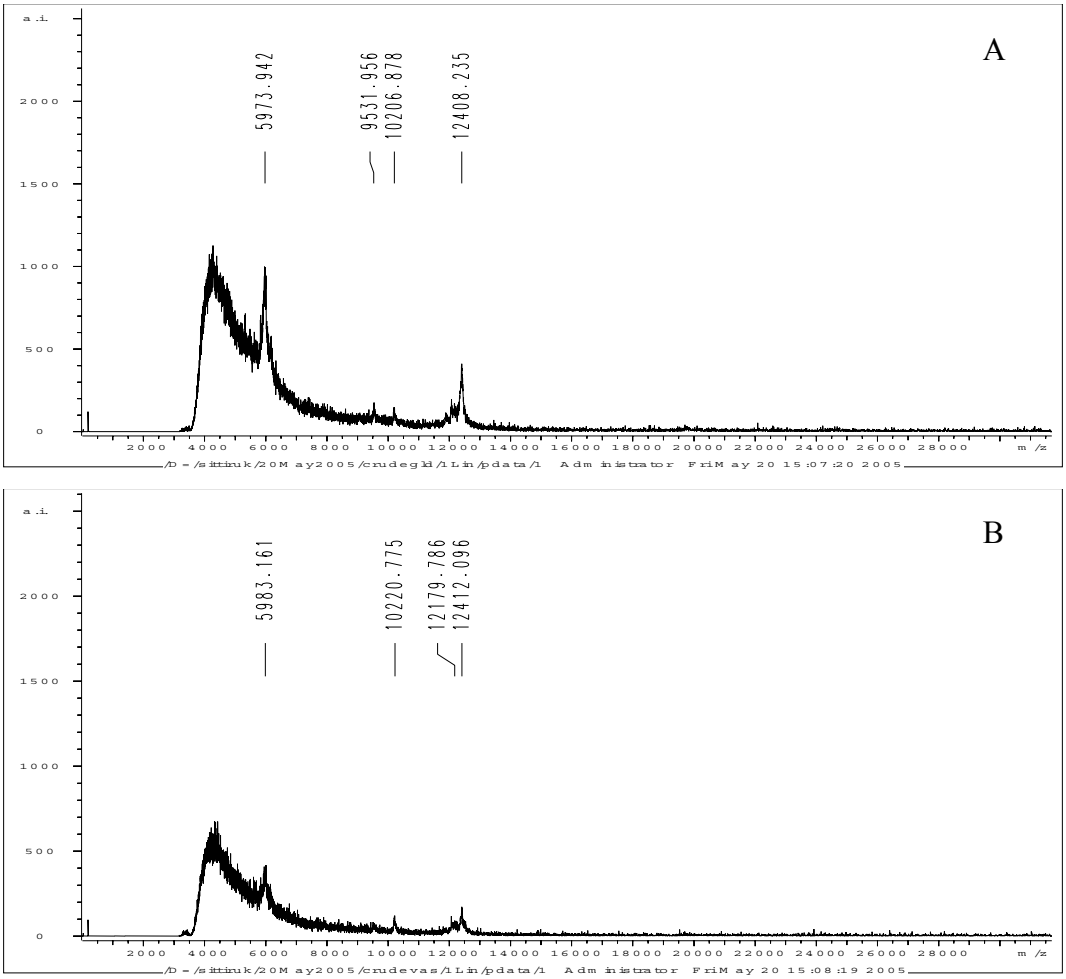


Figure 19 MALDI-TOF MS Spectrum of acetone androgenic tissues (A) and vas deferens (B) extracts

The spectra were recorded in the linear positive ion mode using sinapinic acid matrix. Pooled fractions were collected and evaporated in a vacuum to remove acetonitrile before subject to Bruker Reflex IV matrix assisted laser desorption ionization (MALDI) mass spectrometer equipped with a delayed extraction source and 337 nm pulsed nitrogen laser. One microliter was mixed with 1 μl of 50 mM sinapinic acid and applied to the sample probe. The instrument was run in the linear mode using 20 kV acceleration.

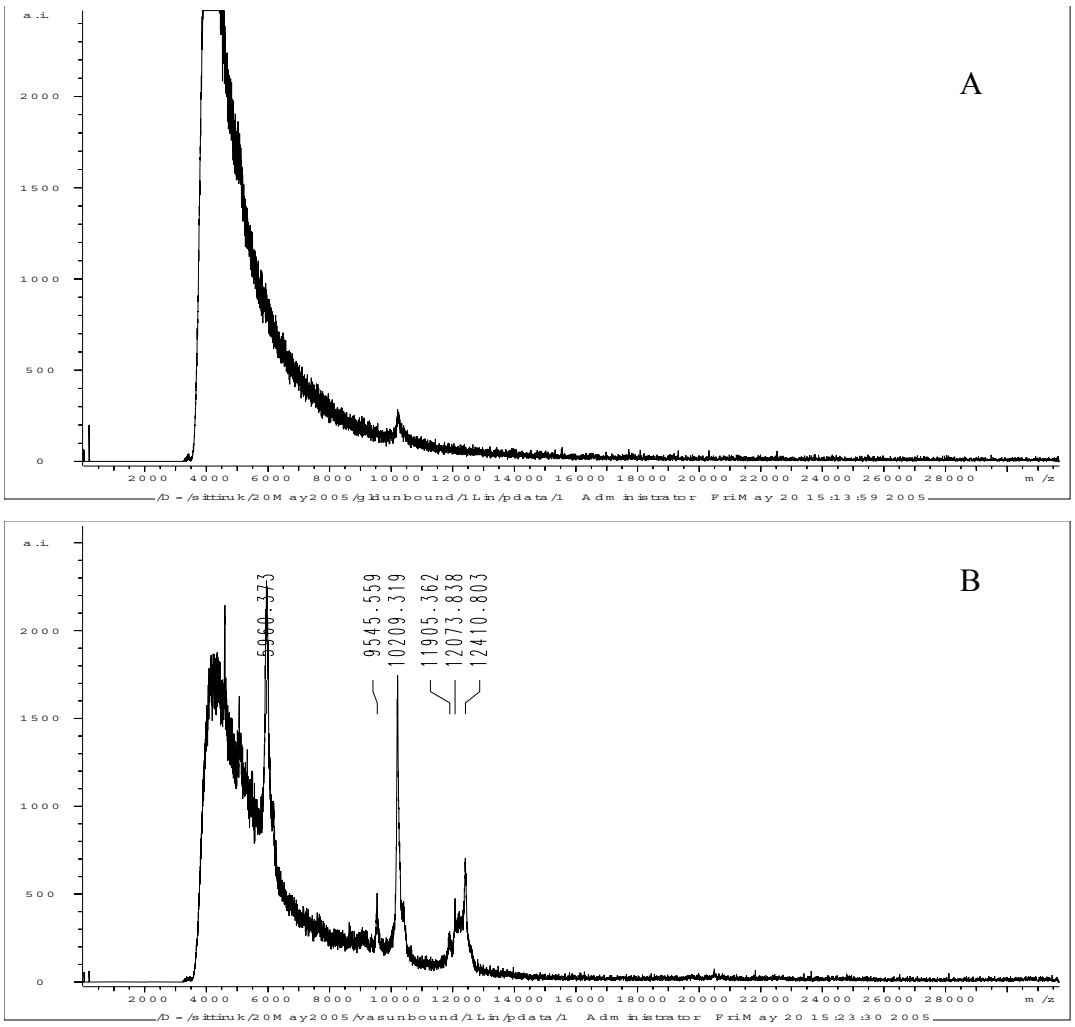


Figure 20 MALDI-TOF MS Spectrum of pooled unbound fractions obtained from RP (Sep-Pak C18 catridge) -HPLC separated acetone androgenic tissues (A) and vas deferens (B) extracts.

The spectra were recorded in the linear positive ion mode using sinapinic acid matrix Pooled fractions were collected and evaporated in a vacuum to remove acetonitrile before subject to Bruker Reflex IV matrix assisted laser desorption ionization (MALDI) mass spectrometer equipped with a delayed extraction source and 337 nm pulsed nitrogen laser. One microliter was mixed with 1 µl of 50 mM sinapinic acid and applied to the sample probe. The instrument was run in the linear mode using 20 kV acceleration.

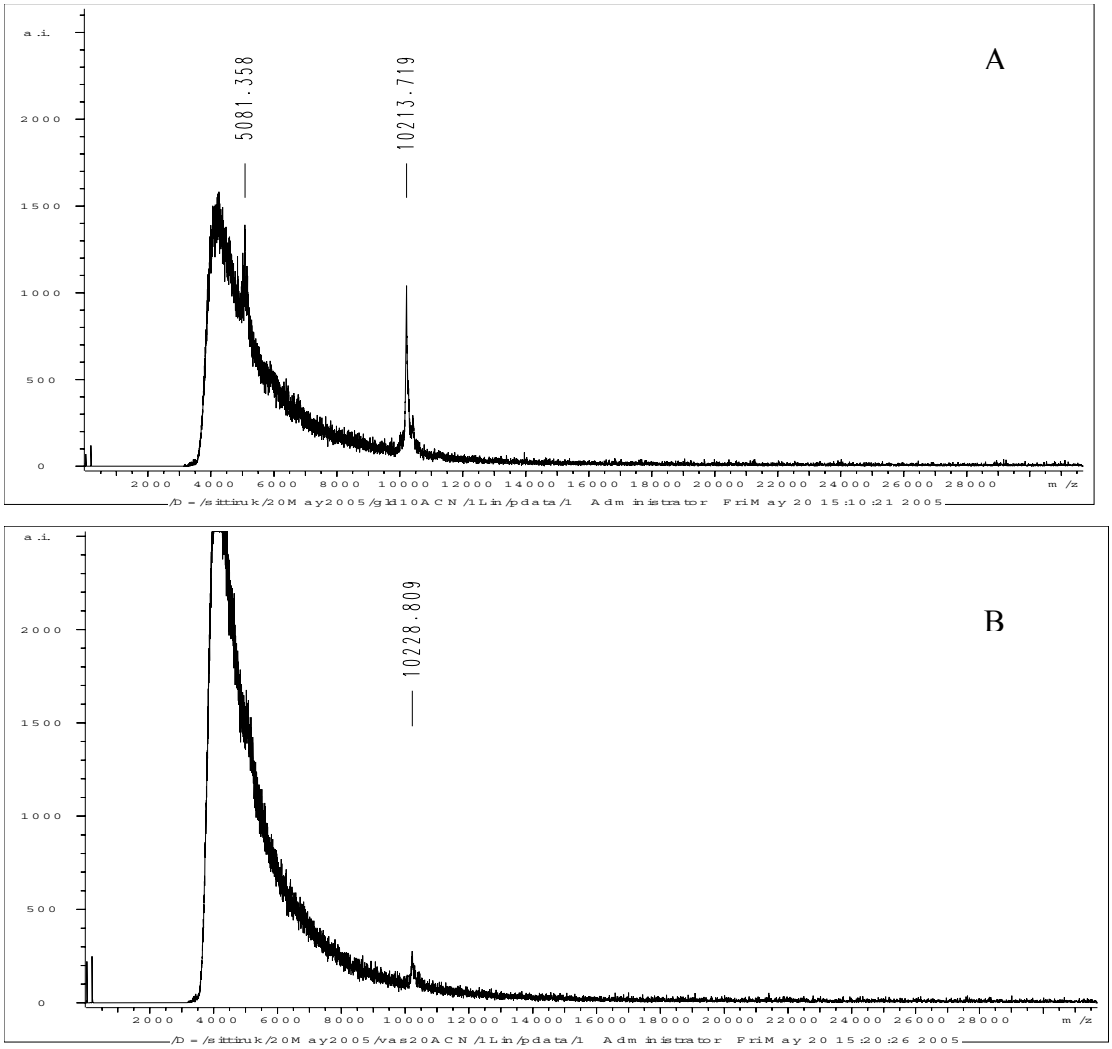


Figure 21 MALDI-TOF MS Spectrum of pooled 20% acetronile fractions obtained from RP(Sep-Pak C18 catridge)-HPLC separated acetone androgenic tissues (A) and vas deferens (B) extracts.

The spectra were recorded in the linear positive ion mode using sinapinic acid matrix. Pooled fractions were collected and evaporated in a vacuum to remove acetonitrile before subject to Bruker Reflex IV matrix assisted laser desorption ionization (MALDI) mass spectrometer equipped with a delayed extraction source and 337 nm pulsed nitrogen laser. One microliter was mixed with 1 µl of 50 mM sinapinic acid and applied to the sample probe. The instrument was run in the linear mode using 20 kV acceleration.

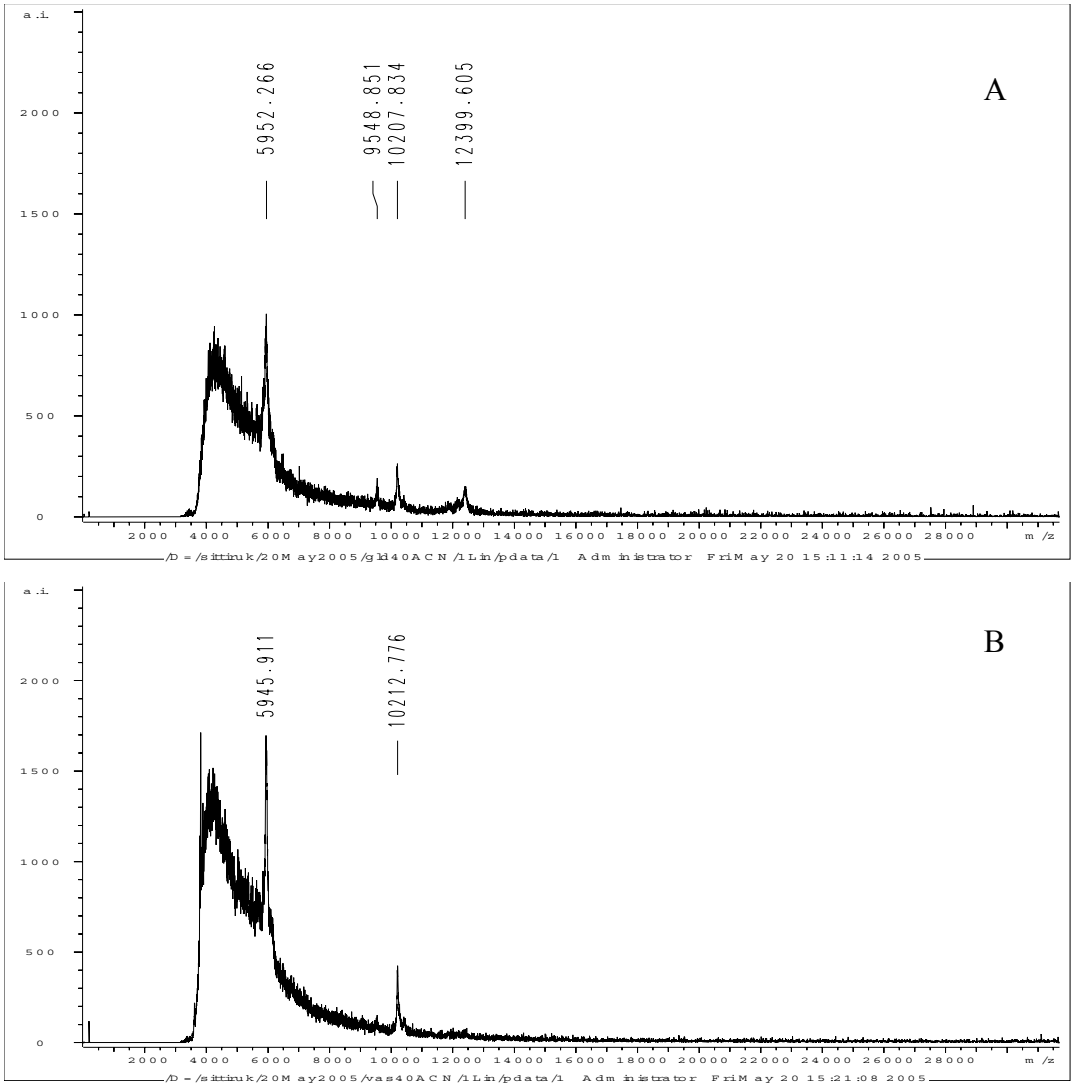


Figure 22 MALDI-TOF MS Spectrum of pooled 40% acetronile fractions obtained from RP(Sep-Pak C18 catridge)-HPLC separated acetone androgenic tissues (A) and vas deferens (B) extracts.

The spectra were recorded in the linear positive ion mode using sinapinic acid matrix. Pooled fractions were collected and evaporated in a vacuum to remove acetonitrile before subject to Bruker Reflex IV matrix assisted laser desorption ionization (MALDI) mass spectrometer equipped with a delayed extraction source and 337 nm pulsed nitrogen laser. One microliter was mixed with 1 μl of 50 mM

sinapinic acid and applied to the sample probe. The instrument was run in the linear mode using 20 kV acceleration.

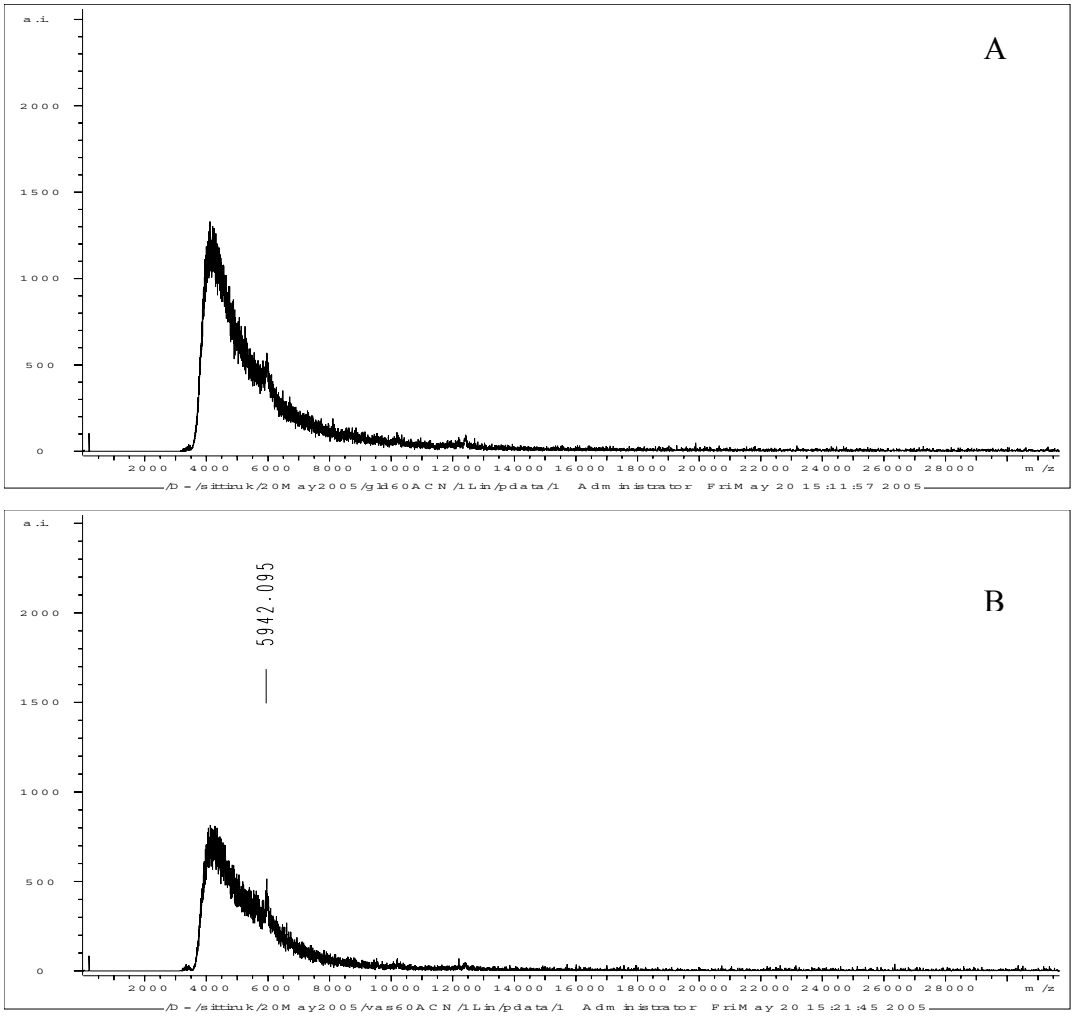


Figure 23 MALDI-TOF MS Spectrum of pooled 60% acetronile fractions obtained from RP(Sep-Pak C18 catridge)-HPLC separated acetone androgenic tissues (A) and vas deferens (B) extracts.

The spectra were recorded in the linear positive ion mode using sinapinic acid matrix. Pooled fractions were collected and evaporated in a vacuum to remove acetonitrile before subject to Bruker Reflex IV matrix assisted laser desorption ionization (MALDI) mass spectrometer equipped with a delayed extraction source and 337 nm pulsed nitrogen laser. One microliter was mixed with 1 µl of 50 mM

sinapinic acid and applied to the sample probe. The instrument was run in the linear mode using 20 kV acceleration.

No product was observed in the RP-HPLC unbound fractions of androgenic tissues (Fig. 19A) which differed from the vas deferens fractions (Fig. 19B) whose molecular masses were read in the range 5.9 – 12.4 kDa. The result implies the different of protein composition in the acetone androgenic tissues and vas deferens extracts. Peak of molecular mass approximately 10.2 kDa was found in 20% and 40% acetronitrile fractions of both the acetone androgenic tissues and vas deferens extracts. However, much more different in the peak height of acetone androgenic tissues extract when compared to those of the vas deferens extract was shown in the 20% acetronitrile fractions.

The acetone angrogenic tissue and vas deferens extracts were re-chromatrographied by reverse phase HPLC, on a Lichrosorb C18 column and the bound products were eluted with a linear gradient of 0-100 % acetonitrile in 0.05% TFA. The RP-HPLC chromatogram displayed complex peaks which were not well resolved as shown in Fig. 24. Each fraction was analyzed by MALDI-TOF MS and it was found that at 42.5% acetronitrile, the fraction no. 22 of the acetone AG extract contained a major protein of molecular mass 10.2 kDa (Fig 25A) while 10.2 kDa spectrum was not found in the fraction no. 22 of the acetone vas deferens extract (Fig 25B). This 10.2 kDa peak is assumed to be the AGH because it was presented only in the androgenic tissues.

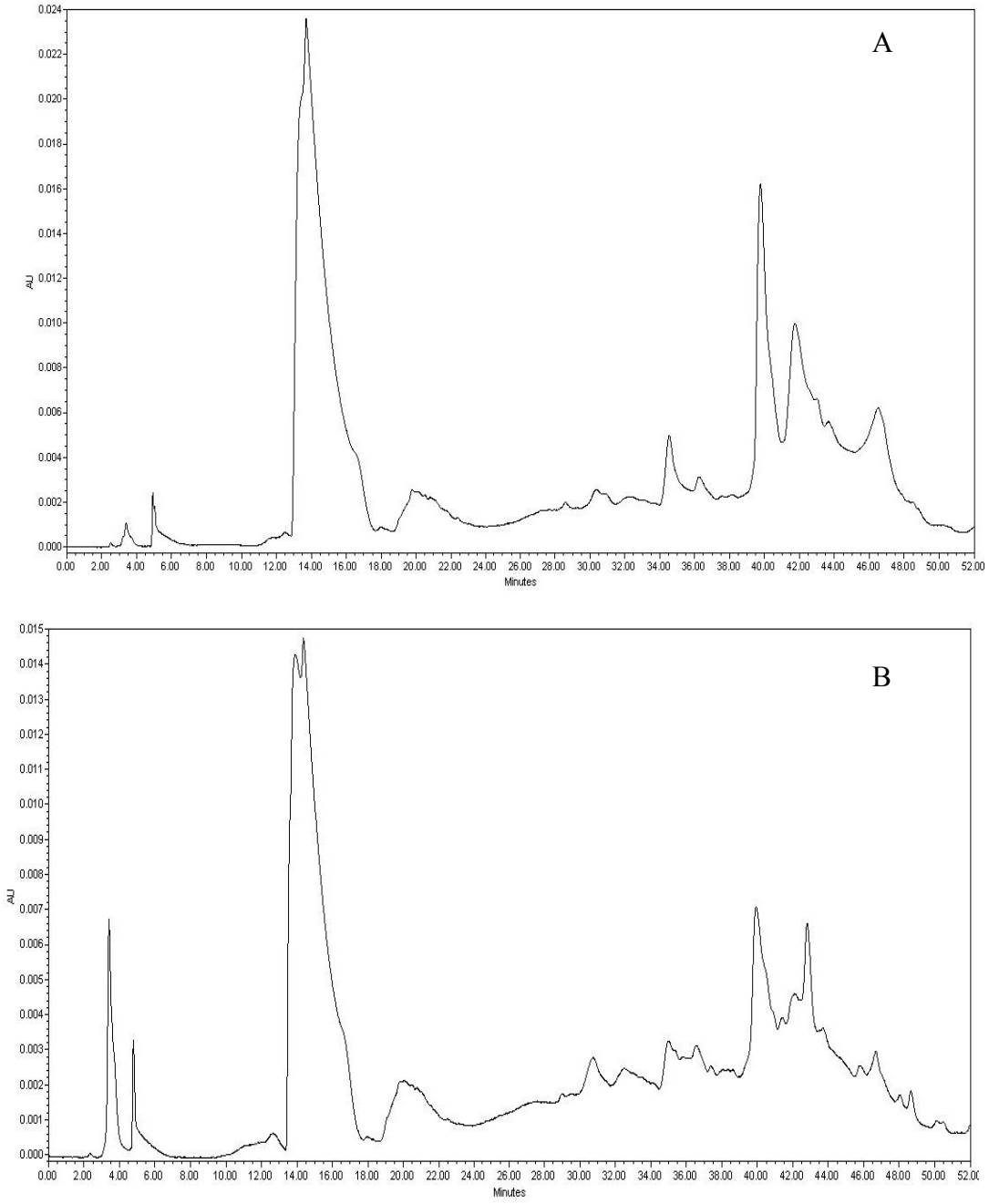


Figure 24 Reverse phase HPLC of acetone extracts of the androgenic tissues (A) and vas deferens (B) on Lichrosorb column.

The acetone androgenic tissues (A) and vas deferens (B) extracts of the giant freshwater prawn (25 µg / 5 µl) were applied to a Lichrosorb column, pre-equilibrated with 0.1% TFA. The column was washed with the equilibration solvent

and the adsorbed materials were gradient eluted with 0-100 % acetonitrile containing 0.1% TFA at a flow rate of 1 mg/ml. Fractions of 1 ml were collected.

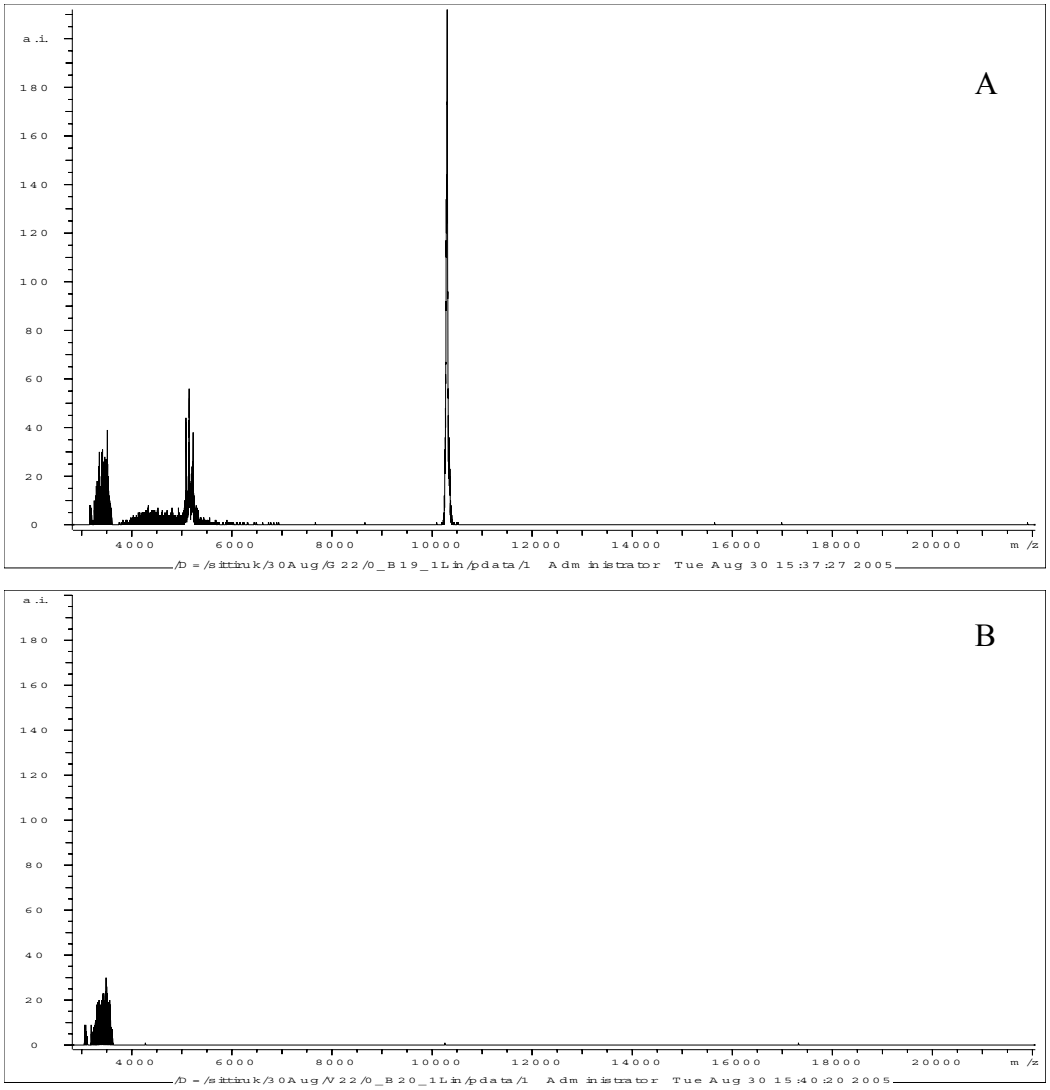


Figure 25 MALDI-TOF MS Spectrum of acetronile fraction no. 22 obtained From RP (Lichrosorb)-HPLC separated acetone androgenic tissues (A) and vas deferens (B) extracts.

The spectra were recorded in the linear positive ion mode using sinapinic acid matrix. Pooled fractions were collected and evaporated in a vacuum to remove acetonitrile before subject to Bruker Reflex IV matrix assisted laser desorption ionization (MALDI) mass spectrometer equipped with a delayed extraction source

and 337 nm pulsed nitrogen laser. One microliter was mixed with 1 μl of 50 mM sinapinic acid and applied to the sample probe. The instrument was run in the linear mode using 20 kV acceleration.

1.3 Characterization of AGH structure

Because there was some reports of androgenic hormone in other crustaceans having one N-glycosylation and two polypeptides which were linked by two disulfide brigdes (Martin *et al.*, 1999; Ohira *et al.*, 2003). In this experiment, the 10.2 kDa RP-HPLC purified protein of androgenic tissues was investigated for its structure using enzymatic deglycosylation by N-Glycosidase F (PNGase F) and reduction of disulfide bridges by dithiothrietol (DTT). Then, the reaction mixtures were analyzed by MALDI-TOF MS. The results obtained in this study did not show the different mass when compared to the mass before deglycosylation and reduction (Figure 26). This implied that the glycan moiety and disulfide bridges between 2 peptide chains were not present on the 10.2 kDa protein.

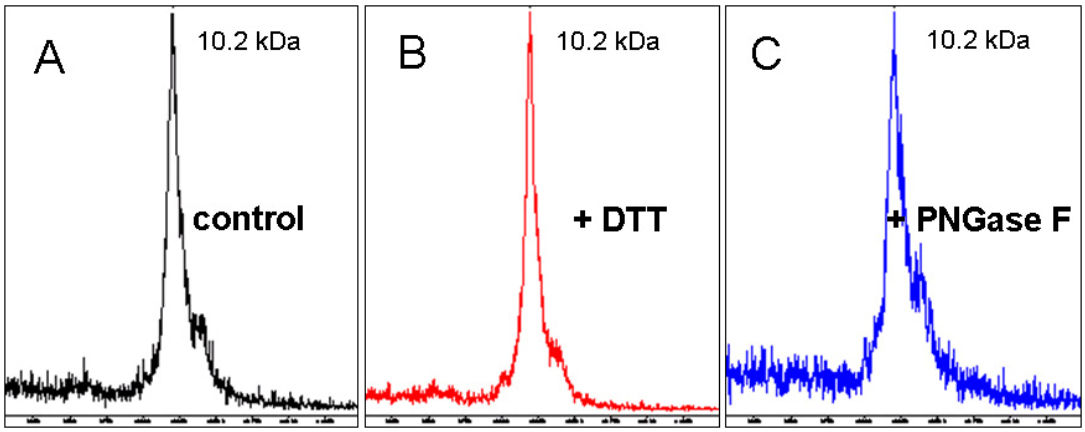


Figure 26 MALDI-TOF MS Spectra of the RP-HPLC 10.2 kDa protein purified from androgenic tissues (A), after reduction of disulfide bridges with DTT (B) and enzymatic deglycosylation with PNGase F (C).

The instrument Bruker Reflex IV MALDI was run in the linear mode using 20 kV acceleration. The 1 µl of control or deglycosylated or reduced proteins were mixed with 1 µl of matrix solution (20 mg/ml of sinapinic acid in acetonitrile/0.1% TFA, 50:50, v/v). Finally, 0.5 µl of the mixture was deposited onto the MALDI as target plate. All spectra are the results of signal averaging of 200 shots.

1.4 Identification of AGH by peptide mass fingerprint using endoproteinase Lys-C digestion

In order to characterize the molecular basis of the RP-HPLC purified 10.2 kDa protein, it was subjected to identify by peptde mass fingerprint. A combination of endoproteinase Lys-C digestion and in silico matching of peptides masses against protein databases was used. The MALDI-TOF MS spectrum of endoproteinase Lys-C digested peptides was shown in Fig. 27. The peptide mass fingerprint analysis by MASCOT search did not show the results matching to AGHs of any crustaceans available in the NCBI database (data not shown).

1.5 Protein analysis by tricine-sodium dodecyl sulfate polyacrylamide gel Electrophoresis

The acetone extracts of androgenic tissues and vas deferens were analyzed for the different in their protein band patterns using 15% tricine-SDS polyacrylamide gel electrophoresis (tricine-SDS-PAGE). Several polypeptide bands at 9.7, 10.7, 12.5 and 13.6 kDa, were detected in both tissues by coomassie blue staining as shown in Fig. 28. These polypeptides were selected to be analyzed by LC/MS. Moreover, the another two protein bands at about 10 and 15 kDa which were seen in the other gel running (data not shown) were also selected to be analyzed.

1.6 *De novo* sequencing of androgenic gland hormone by LC/MS

The 9.7, 10.7, 12.5, and 13.6 kDa proteins from tricine-SDS-PAGE each was in-gel digested by trypsin and the amino acid sequence of tryptic peptides was determined by LC-MS-MS. The internal peptide sequences derived from trypsin digestion were shown in Table 6. All of the possible sequences were used to do similarity search against the amino sequence database in the NCBI, using BlastP program. There was no match with any proteins in the database. Nevertheless, these sequences were used to design some degenerated primers specific to the AGH as shown in Table 3.

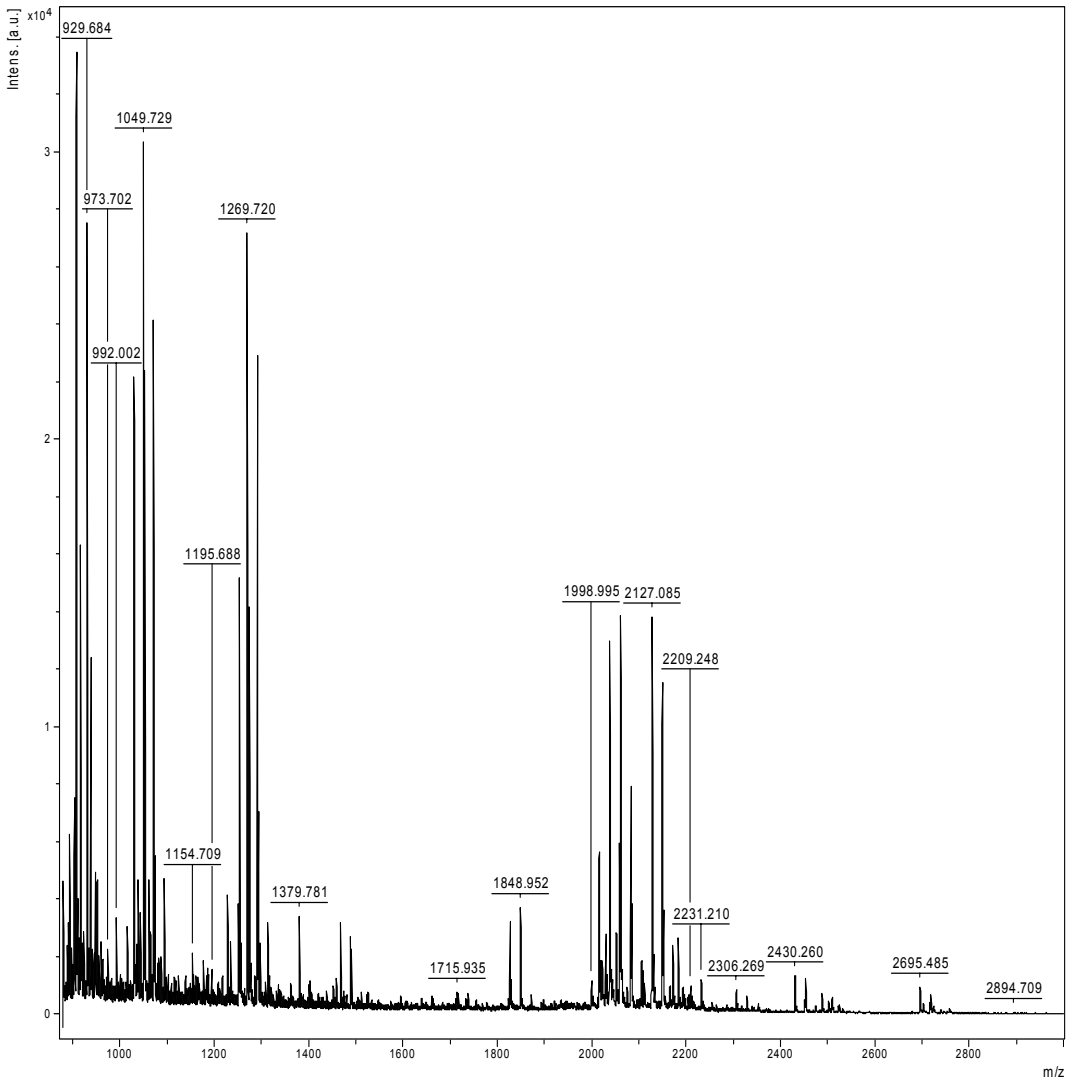


Figure 27 The MALDI-TOF MS Spectrum of peptides from endoproteinase Lys-C digested of RP-HPLC purified 10.2 kDa protein from androgenic tissues.

The digested peptide mixture was mixed with saturated 2,5-dihydroxybenzoic acid (DHBA) matrix solution and spotted onto the MALDI target plate. The instrument Bruker Reflex IV MALDI was run in the refractor mode using 20 kV acceleration.

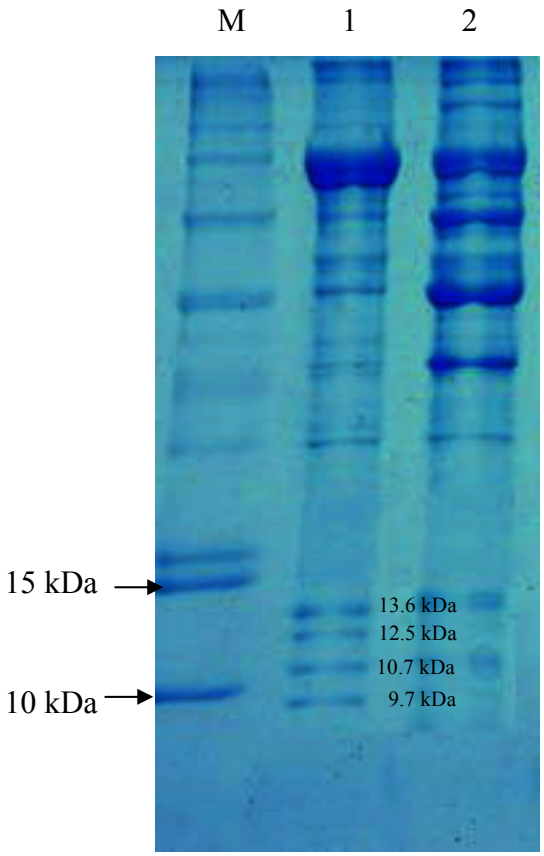


Figure 28 Tricine-SDS-PAGE of acetone extracts of androgenic tissues and vas deferens from freshwater prawn using 15% gel.
Lane1, marker proteins; Lane 2, acetone extract of androgenic tissues (30 µg); Lane 3, acetone extract of vas deferens.

Table 6 Sequences of the peptide fragments derived from tryptic digested peptides of *Machrobrachiun rosenbergii* androgenic tissues analyzed by LC-MS-MS

Protein Size	Peptide MW (Da)	Number of Amino acid	Sequence	Retention Time (min)
15	1282.667	11	(-)ELGAVLDWQPR(-)	27.91
	1282.667	11	(-)LEGAVLDWQPR(-)	27.91
	1395.663	15	(-)SAQSAYEALGAGSGK(-)	24.79
	1395.663	15	(-)ASQSAYEALGAGSGK(-)	24.79
	1395.663	15	(-)TGQSAYEALGAGSGK(-)	24.79
	1395.663	15	(-)SAQSAYEAGLAGSGK(-)	24.79
	1395.663	15	(-)ASQSAYEAGLAGSGK(-)	24.79
	1394.679	15	(-)SAQSAYQALGAGSGK(-)	24.79
	1408.695	16	(-)GVGSSAYQAGLAGSGK(-)	25.78
	1408.695	16	(-)VGGSSAYQAGLAGSGK(-)	25.78
	1408.706	15	(-)RGSSAYQAGLAGSGK(-)	25.78
	1408.695	15	(-)GVGSSAYQQLAGSGK(-)	25.78
	1408.695	16	(-)GVSGSAYQAGLAGSGK(-)	25.78
	1402.721	15	(-)GLSGAAYQGQLGPGK(-)	27.85
	1402.721	15	(-)GLGSAAYQGQLGPGK(-)	27.85
15	1402.721	16	(-)GLSGAAYQGGLAGPGK(-)	27.85
	1402.721	15	(-)AVSGAAYQGQLGPGK(-)	27.85
	1402.721	15	(-)LGGSAAYQGQLGPGK(-)	27.85
	1268.673	13	(-)ELGAVLTGAEGPR(-)	27.91
	1268.673	13	(-)ELGAVLTGEAGPR(-)	27.91
	1268.673	13	(-)LEGAVLTGEAGPR(-)	27.91
	1268.673	12	(-)ELGAVLTQEGPR(-)	27.91
	1268.673	13	(-)ELGAVLTGEAPGR(-)	27.91
	1268.672	13	(-)ELGAVLTGGPEAR(-)	29.09
	1268.672	13	(-)LEGAVLTGGPEAR(-)	29.09
	1268.677	13	(-)ELGAVLTGGPLWG(-)	29.09

Table 6 (Continue)

Protein Size	Peptide MW (Da)	Number of Amino acid	Sequence	Retention Time (min)
10	1065.546	9	(-)VFDETTARK(-)	25.21
	1065.534	9	(-)VFDETTNLK(-)	25.21
	1065.534	9	(-)FVDETTNLK(-)	25.21
	1065.534	10	(-)VFDETTGGLK(-)	25.21
	1065.534	9	(-)VFDETTNKL(-)	25.21
	806.4075	6	(-)LAWDFR(-)	25.3
	1032.524	8	(-)TLLEWESR(-)	25.35
	1032.524	8	(-)LTLEWESR(-)	25.35
	1025.518	8	(-)VSEYLYPR(-)	25.75
	806.4075	6	(-)ALWDFR(-)	25.3
9.7	1226.6367	11	(-)PGSLSNLQQQR(-)	26.58
10.7	807.3763	11	(K)QFDAAEK(A)	18.97
12.5	1407.6517	12	(-)DEDLASEVLDFR(-)	29.98
13.6	887.46	7	(-)ELLEQEK(-)	23.05
	887.46	7	(-)LELEQEK(-)	23.05
	847.4803	8	(-)LGGYPTLK(-)	22.37

2. Molecular cloning of androgenic gland hormone from cDNA

2.1 Cloning and sequencing of the partial cDNA fragments encoding androgenic gland hormone

Total RNA was extracted from androgenic glands from 12 male prawns which has average weight about 100 g per prawn using TRIzol reagent and was used as a template for RT-PCR. Total weight of extracted gland is 211 mg. The isolated total RNA had an A 260/A280 ratio of 1.83 suggesting that the RNA had high quality without any degraded RNA and contaminated genomic DNA. Yield of the total RNA was approximately 0.42 μg per mg tissues. Total RNA was run on 1% agarose gel and the two expected bands of 18s and 28s rRNA were observed as shown in Fig. 29

All PCR products derived from pairing AGHm-F1, AGHm-F2, AGHm-F3, AGHm-R1, AGHm-R2, AGHm-R3 and AGHm-R4 primers (synthesized based on the nucleotide sequence of the AGH from isopods) have the lower size than the expected size. PCR amplification derived from pairing between AGHm-F2/AGHm-R2, AGHm-F3/AGHm-R2, AGHm-F2 /AGHm-R3 and AGHm-F3/ AGHm-R3 gave many bands (Fig. 30A: lane 6, 7, 9 and 10, respectively) which were non-specific products. As same as PCR amplification using AGHm-F4, AGHm-F5, AGHm-R5 and AGHm-R6 primers (synthesized based on the amino acid sequences of *Cherex quadricarinatus* insulin-like androgenic gland factor), there were many non-specific PCR products too. However, PCR products derived from pairing between AGHm-F2/ AGHm-R2 and AGHm-F5 /AGHm-R6 (Fig. 30A: lane 2 and Fig. 30B: lane 5, respectively) gave an interesting band with the nearly expected size. However, The PCR products of the expected size and the PCR products which gave the sharp band were selected to clone into pGEM-T easy and transformed into *E. coli* JM 109 and the nucleotide sequence of each clones was determined.

The nucleotide sequences of the PCR products were compared to AGH from *A. vulgare*, *P. scaber*, *P. dillatatus* and of *C. quadricarinatus* insulin-like androgenic gland factor. There was no similarity among them significantly (data not

shown). However, the deduced amino acid sequences of these products were further analysed.

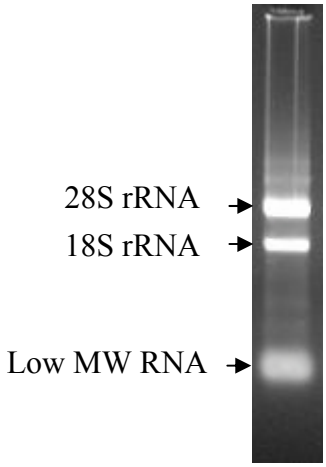
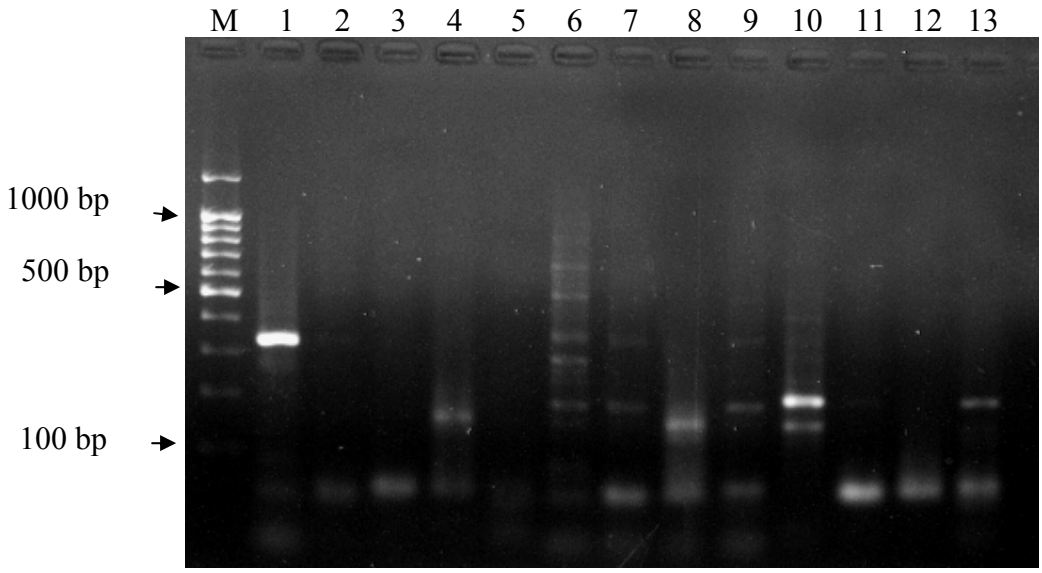


Figure 29 Agarose gel electrophoresis of total RNA from androgenic glands of *M. rosenbergii* for molecular cloning of androgenic gland hormone.

Total RNA was separated by electrophoresed on 1% agarose gel with MOPS buffer at 100 volts for 20 min, stained with ethidium bromide and visualized under UV transilluminator.

Figure 30 (A) PCR fragments amplified by primers based on the nucleotide sequence of the androgenic gland hormone from *Armadillidium vulgare*, *Porcellio scaber* and *P. dillatatus* of 26S rRNA 250 bp. (lane 1: actin as a control (expected sized 337 bp),lane 2: AGHm-F1 and AGHm-R1 (expected sized 110 bp), lane 3: AGHm-F2 and AGHm-R1 (expected sized 110 bp), lane 4: AGHm-F3 and AGHm-R1 (expected sized 50 bp), lane 5: AGHm-F1 and AGHm-R2 (expected sized 130 bp), lane 6: AGHm-F2 and AGHm-R2 (expected sized 130 bp), lane 7: AGHm-F3 and AGHm-R2 (expected sized 70 bp), lane 8: AGHm-F1 and AGHm-R3 (expected sized 299 bp), lane 9: AGHm-F2 and AGHm-R3 (expected sized 299 bp), lane 10: AGHm-F3 and AGHm-R3 (expected sized 239 bp), lane 11: AGHm-F1 and AGHm-R4 (expected sized 327 bp), lane 12: AGHm-F2 and AGHm-R4 (expected sized 327 bp), lane 13: AGHm-F3 and AGHm-R4 (expected sized 267 bp), Lane M: 100 bp ladder plus 1.5 kb)

(B) PCR fragments amplified by primers based on the nucleotide and amino acid sequences of *Cherex quadricarinatus* insulin-like androgenic gland factor. (lane 1: actin as a control (expected sized 337 bp), lane 2: AGHm-F4 and AGHm-R5 (expected sized 349 bp), lane 3: AGHm-F5 and AGHm-R5 (expected sized 292 bp), lane 4: AGHm-F4 and AGHm-R6 (expected sized 421 bp), lane 5: AGHm-F5 and AGHm-R6 (expected sized 364 bp), lane M: 100 bp ladder plus 1.5 kb)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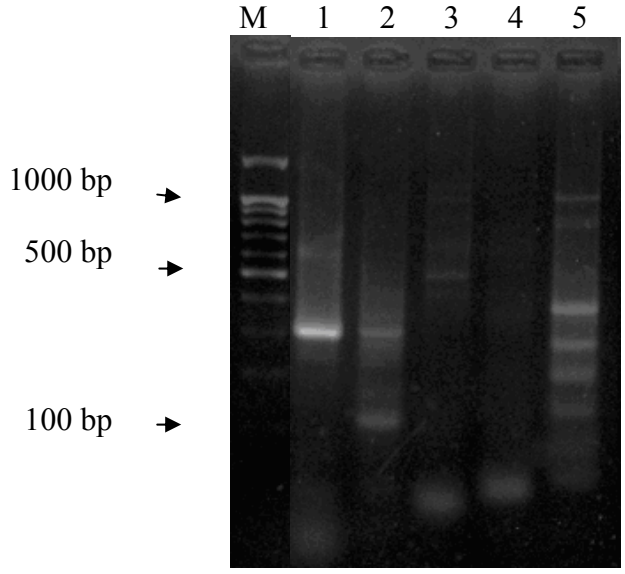
1000 bp
500 bp
100 bp

A

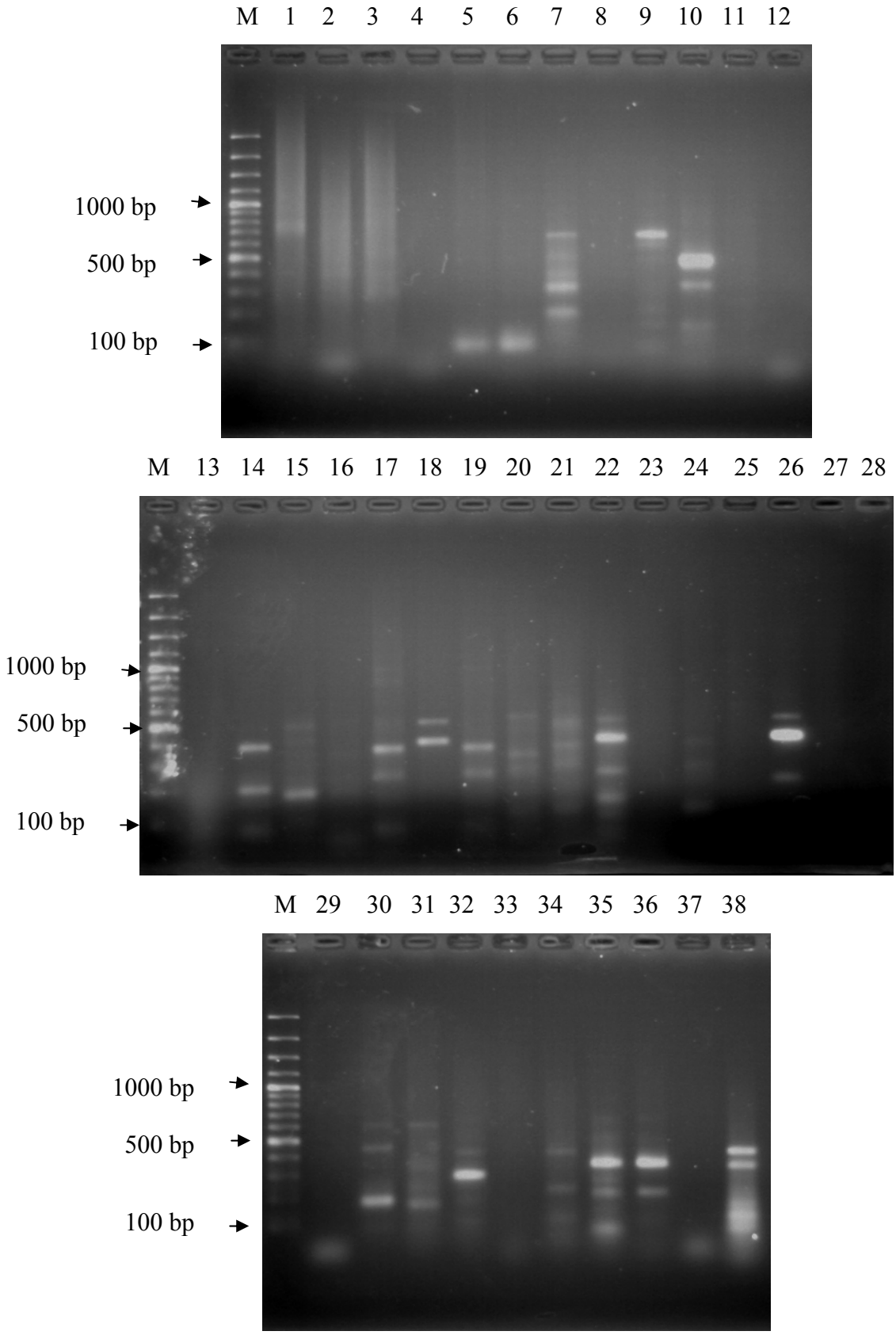

M 1 2 3 4 5
1000 bp
500 bp
100 bp

B

The result of the amplification using the degenerated primer designed from amino acid sequences of 9.7, 10.7, 12.5, 13.6, 10, and 15 kDa peptide fragments (10D_F1, 10D_F2, 10D_F3, 10D_F4, 10D_RF1, 10D_R2, 10D_R3, 10D_R4, 15D2_F1, 15D2_F2, 15D2_F3, 15D2_R1, 15D2_R2, 15D2_R3, AG9.7_F1, AG10.7_F1, AG12.5_F1, AG13.8_F1, AG13.8_F2, AG13.8_F3, AG13.8_R1, AG13.8_R2 and AG13.8_R3) was shown in the Fig. 31. PCR procedure was as described above. There were many non-specific PCR products. Some pairs of primers gave more than one band and some did not give any band. All of PCR products derived from AG13.8_F2/AG13.8_R3, AG13.8_F3 /AG13.8_R1, 10D_F1/10D_R2, 10D_F2/10D_R1, 10D_F2/10D_R2, 10D_F3/10D_R2, 15D2_F2/15D2_R1, 15D2_F3/15D2_R1 and 15D2_F3/15D2_ R2 (lane 9, 10, 14, 17, 18, 22, 26, 32, 35 and 36) which gave sharp and intense band were selected to clone into pCR 8 and transform into *E. coli* Mach1 -T1 and the nucleotide sequence of each clones was determined.

The nucleotide sequences of the PCR products were compared to AGH from *A. vulgare*, *P. scaber*, *P. dillatatus* and of *C. quadricarinatus* insulin-like androgenic gland factor. There was no similarity among them significantly (data not shown). the The nucleotide sequences of these products were further analysed by BlastX. However, they did not share significant sequence homology with any known sequence in the databases.

Figure 31 PCR fragments amplified by primers based on the degenerated primers designed from amino acid sequences of 9.7, 10.7, 12.5, 13.6, 10, and 15 kDa peptide fragments. (Lane 1: AG9.7_F1 and oligo d(T), Lane 2: AG10.7_F1 and oligo d(T),Lane 3: AG12.5_F1 and oligo d(T) Lane 4: AG13.8_F1 and AG13.8_R1, Lane 5: AG13.8_F1 and AG13.8_R2, Lane 6: AG13.8_F1 and AG13.8_R3, Lane 7: AG13.8_F2 and AG13.8_R1, Lane 8: AG13.8_F2 and AG13.8_R2, Lane 9: AG13.8_F2 and AG13.8_R3, Lane 10: AG13.8_F3 and AG13.8_R1, Lane 11: AG13.8_F3 and AG13.8_R2, Lane 12: AG13.8_F3 and AG13.8_R3, Lane 13: 10D_F1 and 10D_R1, Lane 14: 10D_F1 and 10D_R2, Lane 15: 10D_F1 and 10D_R3, Lane 16: 10D_F1 and 10D_R4, Lane 17: 10D_F2 and 10D_R1, Lane 18: 10D_F2 and 10D_R2, Lane 19: 10D_F2 and 10D_R3, Lane 20: 10D_F2 and 10D_R4, Lane 21: 10D_F3 and 10D_R1, Lane 22: 10D_F3 and 10D_R2, Lane 23: 10D_F3 and 10D_R3, Lane 24: 10D_F3 and 10D_R4, Lane 25: 10D_F4 and 10D_R1, Lane 26: 10D_F4 and 10D_R2, Lane 27: 10D_F4 and 10D_R3, Lane 28: 10D_F4 and 10D_R4, Lane 29: 15D2_F1 and 15D2_R1, Lane 30: 15D2_F1 and 15D2_R2, Lane 31: 15D2_F1 and 15D2_R3, Lane 32: 15D2_F2 and 15D2_R1, Lane 33: 15D2_F2 and 15D2_R2, Lane 34: 15D2_F2 and 15D2_R3, Lane 35: 15D2_F3 and 15D2_R1, Lane 36: 15D2_F3 and 15D2_R2, Lane 37: 15D2_F3 and 15D2_R3, Lane 38: actin as control)


M 1 2 3 4 5 6 7 8 9 10 11 12
1000 bp
500 bp
100 bp
M 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
1000 bp
500 bp
100 bp
M 29 30 31 32 33 34 35 36 37 38
1000 bp
500 bp
100 bp

2.2 Androgenic gland cDNA library construction

Total RNA was extracted from androgenic glands from 9 prawns which had average weight about 100 g per prawn. The total weight of 18 glands was 180 mg. The isolated total RNA had an A 260/A280 ratio of 2.17 suggesting that the RNA had high quality. Total RNA was 38 µg and yield of the RNA was approximately 0.42 µg total RNA per mg of tissues. Total RNA was run on 1% agarose gel and the two expected bands of 18s and 28s rRNA were observed (Fig. 32)

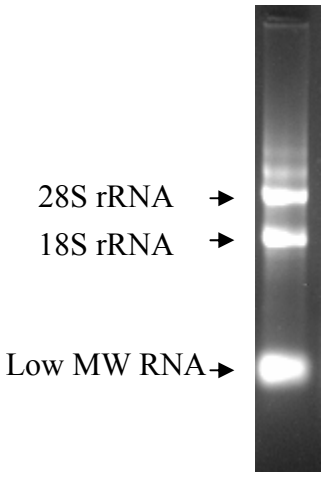


Figure 32 Agarose gel electrophoresis of total RNA from androgenic glands of *M. rosenbergii* for androgenic gland hormone cDNA library construction. Total RNA was separated by electrophoresed on 1% agarose gel with MOPS buffer at 100 volts for 20 min, stained with ethidium bromide and visualized under UV transilluminator.

Messenger RNA was purified from the total RNA of androgenic of *M. rosenbergii* by NucleoTrap mRNA Purification Kits (MACHEREY-NAGEL). The 98.4 ng of mRNA was obtained from 38 µg total RNA. Messenger RNA isolated from total RNA of giant freshwater prawn androgenic gland was subjected for cDNA synthesis and cDNA library construction. Messenger RNA run on 1% agarose gel had good quality with no degradation (Fig. 33)

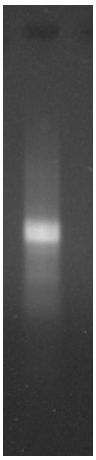


Figure 33 Agarose gel electrophoresis of messenger RNA from androgenic glands of *M. rosenbergii* for androgenic gland hormone cDNA library construction. Messenger Total RNA was separated by electrophoresed on 1% agarose gel with MOPS buffer at 100 volts for 20 min, stained with ethidium bromide and visualized under UV transilluminator.

Fifty micrograms of messenger RNA was then used as a starting material template for *M. rosenbergii* AGH cDNA library construction. The second-strand cDNA was separated by CHROMA SPIN-400 column to fractionate the appropriate cDNA size for further library construction. (Fig. 34) The first four fractions containing cDNA (fractions number 6, 7, 8 and 9) were collected and pooled. The fractionated full-length cDNAs were directionally cloned into TriplEx2 phagemid and transducted into *E. coli* XL1-B.

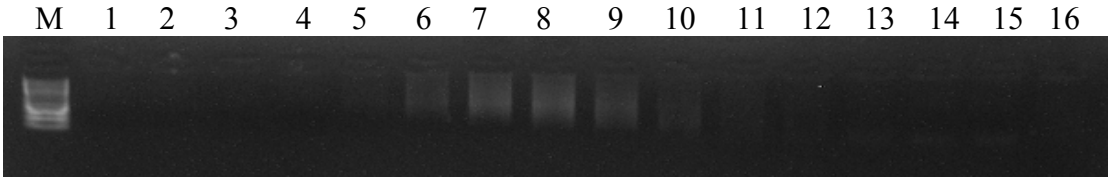


Figure 34 Double-strand cDNA fractionated by CHROMA SPIN-400 column separated by electrophoresed on 1.1% agarose gel with MOPS buffer at 100 volts for 5 min. Lane M is 100 bp ladder. The fractions number 6, 7, 8 and 9 containing cDNA were collected and pooled.

An unamplified library was established by setting up three dilution of unamplified λ lysate and each dilution was tittered by calculation the number of plaque formation. The unamplified library information was shown in Table 7. The 1:5 and 1:10 dilution of unamplified λ lysate had 3 and 3.13 x 10^6 pfu/ml respectively which were acceptable for library construction. Percentage of recombinant clones from blue/white colony calculation was 99%. The 1:10 λ lysate dilution was selected to continue conversion from plaque to colony. The converted BM25.8 colonies were randomly picked up to determining the insert by colony PCR using SP6 and T7 primers. The products of colony PCR were checked on 2% agarose gel as shown in Fig. 35. DNA marker is 100 bp ladder plus 1.5 kb.

Table 7 The number of colony and the titer of 1:5, 1:10 and 1:20 unamplified λ diluted lysate

Dilution factor of Unamplified λ lysate	1:5	1:10	1:20
Number of colony	600	313	50
Titer (10^6 pfu/ml)	3	3.13	1

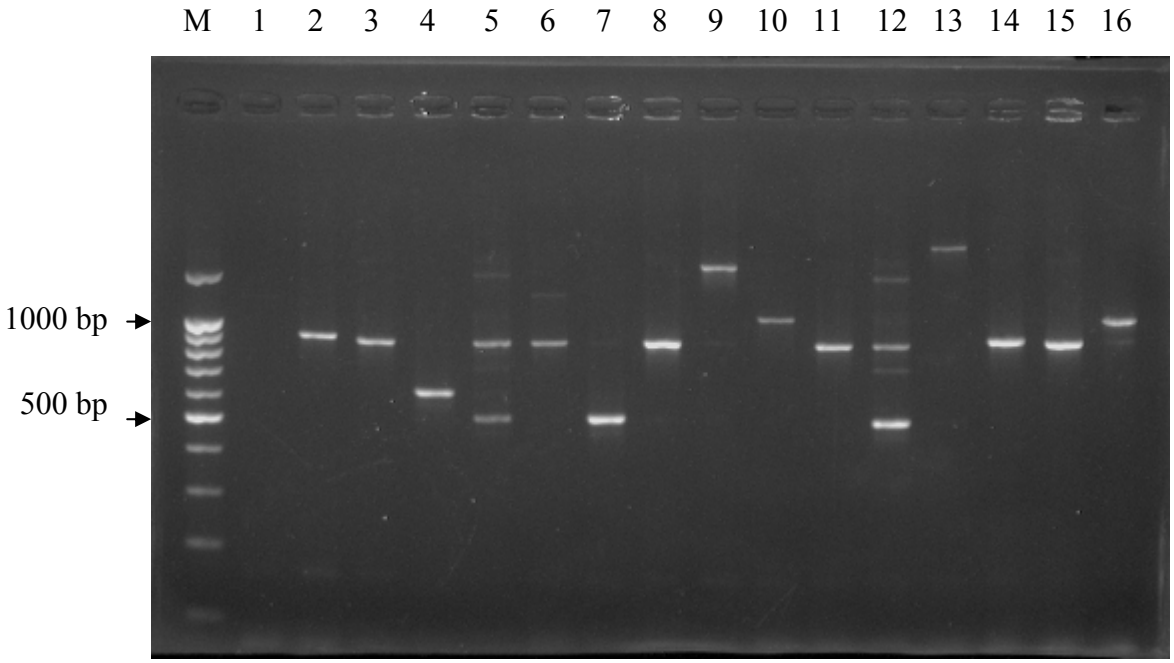


Figure 35 PCR products from colony PCR using SP6 and T7 primers separated by electrophoresed on 2% agarose gel with TAE buffer at 100 volts for 30 min. Lane M is 100 bp ladder.

The total number of colonies derived from an unamplified *M. rosenbergii* androgenic gland cDNA library was 1,100 colonies. A total of 1,000 clones were randomly picked up and purified the plasmids. Then, 960 clones were subjected to single-pass DNA sequencing, resulting in 776 (78%) EST sequences. The average read-length after vector clipping was 500 bp (in the range of 10-777 bp). The very short sequences (less than 50 bp) were not accepted. Then, 766 clones were further analysed. For identification of *M. rosenbergii* cDNA clones, the cutoff was set at an expected value ($E \leq 10^{-4}$). Identified clones generally shared a sequence identity >50% over a relatively long range >150 bp with the most similar sequence from BLASTX. Based on subjective criteria for gene identification at a minimum amino acid sequence similarity of expected value ($E \leq 10^{-4}$), 422 (55%) *M. rosenbergii* transcripts showed homology to previously described genes from *M. rosenbergii* and other species, including shrimp (Table 8). Five clones matching to the vector sequences were sorted out due to the non recombinant clone. ESTs matching known genes were categorized into seven categories on the basis of general functions

(storage protein 30.6%, gene/protein expression and modification 10.1%, hypothetical proteins 9.6%, cell/organism defense 8.3%, enzyme 2.1%, structural protein, cytoskeleton 0.5%, transport proteins 0.5% and other 3.3%) as shown in Fig. 36. In total, 42 known genes were represented and the percentage of transcripts in each category was calculated. Several transcripts matched previously-reported known proteins of *M. rosenbergii*, including alpha-2-macroglobulin, cortical rod-like protein and ferritin.

A total of 266 transcripts did not share significant sequence homology with any known sequence in the databases. Most of them are expected to be AGH or novel genes. Thus, the rests of no significant similarity sequences and some hypothetical proteins with unknown function were further analyzed by translating to protein sequences. After translation, some deduced amino acid sequences were too short to identify the protein and some could not present the start codon or frequently presented the stop codon. These sequences were not further considered. Therefore, the deduced amino acid sequences of 12 clones named B-H07, C-A010, E-A04, E-C04, E-H07, G-C011, I-C05, J-D03, A-D05 (CG31997-PA Tribolium), A-E05 (similar to CG17652-PA Tribolium castaneu) and F-D010 (carcinin-like protein) were further considered by SMART and multiple alignment with the pro-*Cq-IAG*, three pro-AGHs known in isopods (Manor *et al*., 2007; Ohira *et al*., 2003; Okuno *et al*., 1997) and internal peptide sequences from *de novo* sequencing were performed by ClustalW.

Table 8 The summary of *M. rosenbergii* androgenic gland cDNA library sequencing

Product	Species	Number of clones	E-value
No significant similarity found	-	266	-
26S protease regulatory subunit 6A	*Culex pipiens quinquefasciatus*	1	1.00E-89
26S proteasome regulatory complex subunit p50	*Drosophila melanogaster*	2	3.00E-95
40S ribosomal protein S23	*Dermacentor variabilis*	5	6.00E-76
40S ribosomal protein S27E	*Homarus americanus*	1	5.00E-43
60S ribosomal protein L30	*Lycosa singoriensis*	2	4.00E-45
60s ribosomal protein L39	*Ornithodoros parkeri*	1	4.00E-13
AGAP008475-PA	*Anopheles gambiae*	1	2.00E-46
alpha-2-macroglobulin	*Macrobrachium rosenbergii**	1	1.00E-44
anti-lipopolysaccharide AA-K isoform	*Litopenaeus vannamei**	1	1.00E-09
anti-lipopolysaccharide factor isoform 1	*Farfantepenaeus paulensis**	1	2.00E-25
anti-lipopolysaccharide factor like protein	*Marsupenaeus japonicus**	3	4.00E-25
cadmium metallothionein	*Palaemonetes pugio**	226	6.00E-04
calpain T	*Gecarcinus lateralis***	2	6.00E-107
carcinin-like protein	*Carcinus maenas***	4	2.00E-14
cathepsin D	*Penaeus monodon**	1	6.00E-107
Cofilin/actin-depolymerizing factor homolog (Protein D61) (Protein twinstar)	*Drosophila yakuba*	1	2.00E-14
conserved hypothetical protein	*Culex pipiens quinquefasciatus*	1	6.00E-107
copii-coated vesicle membrane protein P24	*Aedes aegypti*	1	2.00E-14
cortical rod-like protein	*Macrobrachium rosenbergii**	1	6.00E-107

Table 8 (Continued)

Product	Species	Number of clones	E-value
crustin	*Farfantepenaeus brasiliensis**	52	2.00E-14
cyclophilin-like protein	*Phlebotomus papatasi*	1	7.00E-65
cytochrome c oxidase polypeptide IV	*Bombyx mori*	2	2.00E-57
elongation factor-1 alpha	*Libinia emarginata***	3	6.00E-88
Eukaryotic initiation factor 1A	*Drosophila melanogaster*	3	4.00E-38
ferritin	*Macrobrachium rosenbergii**	7	1.00E-18
GA18544 gene product from transcript GA18544-RA	*Drosophila pseudoobscura*	1	8.00E-19
gamma subunit isoform 1	*Mus musculus*	1	4.00E-18
hypothetical protein	*Aedes aegypti*	2	1.00E-07
hypothetical protein	*Thermobia domestica*	23	9.00E-12
hypothetical protein CpipJ_CPIJ000746	*Culex pipiens quinquefasciatus*	16	4.00E-04
hypothetical protein LOC100119759	*Nasonia vitripennis*	13	4.00E-07
hypothetical protein LOC686222	*Rattus norvegicus*	2	1.00E-11
hypothetical protein LOC765150	*Strongylocentrotus purpuratus*	1	3.00E-13
intermediate filament protein C1	*Branchiostoma floridae*	1	7.00E-04
mitochondrial ATP synthase lipid binding protein	*Culex pipiens quinquefasciatus*	1	4.00E-07
mitochondrial cytochrome c oxidase subunit 6B	*Drosophila simulans*	3	3.00E-26
Mitochondrial Transcription Factor B1	*Drosophila melanogaster*	5	9.00E-12
peptidyl-prolyl cis-trans isomerase	*Aedes aegypti*	3	3.00E-64
PREDICTED: similar to CG17652-PA	*Tribolium castaneum*	3	6.00E-29
PREDICTED: similar to putative ribosomal protein L19e	*Strongylocentrotus purpuratus*	2	1.00E-26
putative ribosomal protein L27Ae	*Diaphorina citri*	3	1.00E-53

Table 8 (Continued)

Product	Species	Number of clones	E-value
Putative RNA polymerase II transcriptional coactivator	*Tetrahymena thermophila*	1	9.00E-18
putative serine proteinase inhibitor	*Pacifastacus leniusculus**	2	3.00E-24
ribosomal protein L10A	*Bombyx mori*	1	7.00E-86
ribosomal protein L14	*Gallus gallus*	1	4.00E-30
ribosomal protein L23	*Epinephelus coioides*	3	3.00E-62
ribosomal protein L44e	*Georissus* sp. APV-2005	2	1.00E-40
ribosomal protein LP1	*Argas monolakensis*	2	5.00E-21
ribosomal protein S15	*Danio rerio*	2	3.00E-36
ribosomal protein S15	*Poecilia reticulata*	1	3.00E-14
ribosomal protein S15	*Oncorhynchus masou formosanus*	1	7.00E-10
ribosomal protein S16	*Gallus gallus*	1	5.00E-66
ribosomal protein S27a	*Canis lupus familiaris*	1	1.00E-06
RNA polymerase small Zn-binding subunit,	*Aedes aegypti*	6	4.00E-18
SEC61, gamma subunit	*Mus musculus*	3	4.00E-18
similar to 60S ribosomal protein L36	*Ornithorhynchus anatinus*	1	2.00E-25
similar to apontic CG5393-PB, isoform B	*Apis mellifera*	1	8.00E-07
similar to ATPase inhibitor-like protein	*Nasonia vitripennis*	21	6.00E-15
similar to CG14235-PA, isoform A	*Tribolium castaneum*	7	2.00E-26
similar to Glycine cleavage system H protein,mitochondrial precursor (Pumpless protein)		1	4.00E-18

Table 8 (Continued)

Product	Species	Number of clones	E-value
similar to nudix (nucleoside diphosphate linked moiety X)-type motif 21	*Apis mellifera*	1	1.00E-98
similar to ornithine decarboxylase	*Nasonia vitripennis*	1	4.00E-21
similar to prefoldin 1	*Tribolium castaneum*	1	1.00E-05
similar to putative ribosomal protein L19e	*Strongylocentrotus purpuratus*	10	1.00E-26
similar to ribosomal protein S5	*Apis mellifera*	4	2.00E-65
similar to SCCA2/SCCA1 fusion protein	*Equus caballus*	1	1.00E-05
similar to ubiquinol-cytochrome c reductase complex7.2kDa protein isoform a	*Tribolium castaneum*	1	2.00E-12
SUMO-1-like protein	*Artemia franciscana*	5	3.00E-37
tail muscle elongation factor 1 gamma	*Procambarus clarkii*	2	2.00E-76
threonyl-tRNA synthetase	*Aedes aegypti*	1	3.00E-37
translation initiation factor 4C (1A)	*Anopheles gambiae*	1	2.00E-37
transmembrane protein 14C	*Bombyx mori*	1	3.00E-30
ubiquitin	*Portunus pelagicus***	1	2.00E-60
unnamed protein product	*Tetraodon nigroviridis*	1	1.00E-37
vitamin K epoxide reductase complex subunit 1 precursor	*Ixodes scapularis*	2	2.00E-36
Total		761	

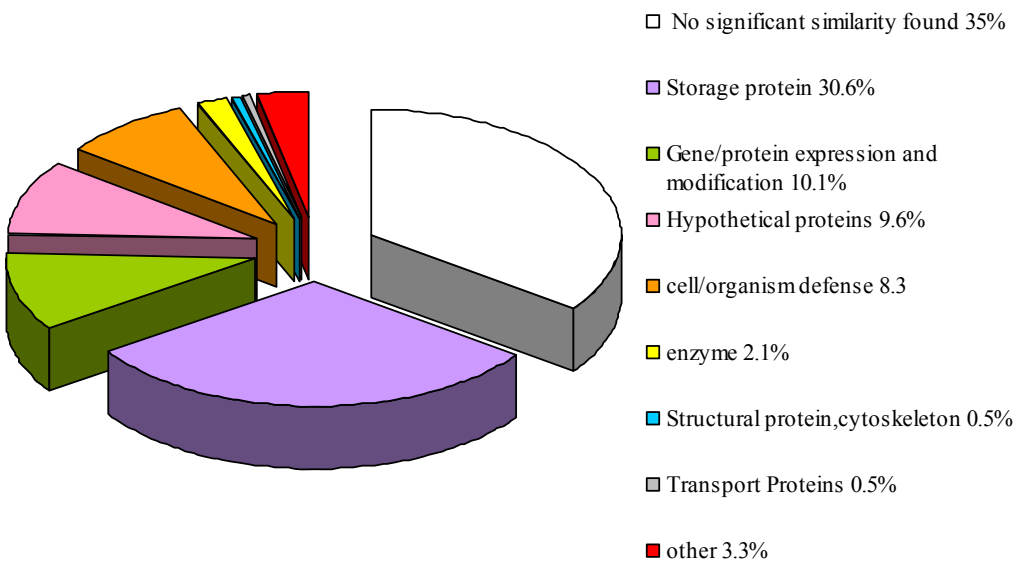


Figure 36 Sequence similarity analysis of 761 ESTs (61 putative genes) from *M. rosenbergii* androgenic gland cDNA library by BLASTX. More than 35% of the sequences had no significant similarity (E-value > 0.0001) to any Uniprot protein. 9.6% were similar to hypothetical proteins, and the rest could be related to proteins associated with protein in the NCBI database.

The SMART domain search tool predicted the existence of a domain between residues 22-84 and 27-71 of C-A010 similar to insulin growth factor-binding protein (E value= 2.46e+03) and to Laminin-type epidermal growth factor-like domain (E-value = 9.44e+02), respectively. The residues 24-64 and 66-105 of F-D010 were similar to insulin growth factor-binding protein (E value= 3.39e+03) and to insulin/insulin-like growth factor/relaxin family (E-value = 2.17e+03), respectively (Fig. 37). The deduced amino acids and theoretical molecular weight of C-A010 and F-D010 calculated by ProtParam program (ExPASy Proteomics Server) are 134 amino acids/15 kDa and 111 amino acids /12.2 kDa, respectively. However, there was no deduced amino acid sequence from any clone which ClustalW matching score with the pro-*Cq-IAG*, three pro-AGHs known in isopods and internal peptide sequences from *de novo* sequencing was higher than 20%. Besides that, the deduced amino acid sequence derived from products of primers designed from *A. vulgare*, *P. scaber*, *P.*

dillatatus AGH and of *C. quadricarinatus* insulin-like androgenic gland factor were no similarity to any deduced amino acid sequence from 12 library clones and internal amino acid sequences from de novo sequencing significantly.

Since F-D010 was similar to insulin/insulin-like growth factor/relaxin family which included AGH, the structure of F-D010 deduced amino acid sequence was determined by prediction from bioinformatic tools. The SignalP 3.0 result showed the existing of 16-amino acids signal peptide with max cleavage site probability 0.507 while TargetP 1.1 result revealed that this signal peptide involved the secretory pathway with reliability class 2 (almost strongest prediction). The predicted signal peptide sequence of F-D010 has some sequence similarity to the *A. vulgary*, *P. scaber* and *P. dillatatus* AGH and *Cq-IAG* signal peptides (25, 12, 12 and 6%, respectively) No site of N-glycosylation was predicted in this sequence. There were 12 Cys residues in their sequences which were no prediction of disulfide bond forming.

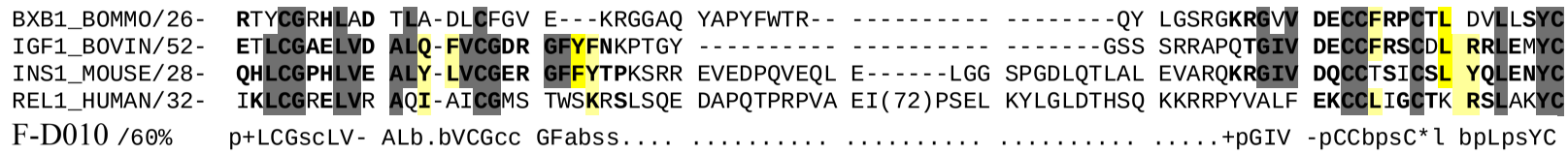


Figure 37 Multiple sequence alignment of the residues 66-105 of F-D010 with representative members of the insulin/insulin-like growth factor/relaxin family, by SMART domain search. The sequences shown include: mouse insulin [INS1_MOUSE, (Wentworth *et al*., 1986)], insulin-like growth factor I of bovine [IGF1_BOVIN, (Fotsis *et al*., 1990)], human relaxin H1 [REL1_HUMAN, (Hudson *et al*., 1984)], bombyxin C-1 of *B. mori* [Silk moth, BXA6_BOMMO, (Nagasawa *et al*., 1986)], Conserved Cys residues are shown in boxes while the (-) indicates gaps introduced into the amino acid sequence to allow for the maximal degree of identity in the alignment.

3. Androgenic gland hormone bioactivity determination

Two microliters of the acid-alcohol fraction, acetone extract of the androgenic tissues and each pooled fraction from Sep-Pak C18 column were injected into 20-30 days old *M. rosenbergii* females at the position of the first ventrally abdominal segments by microsyringe. Eight injections were given, each at 10 day intervals, with the prawns being sacrificed ten days after the final injection. After 80 days of injections, the animals were observed for indications of masculinization or inhibition of feminine trait. The result of bioactivity determination was presented in Table 9

Table 9 Survival of the musculinized *Macrobrachium rosenbergii* at 80 days after the injection

group	Sample of injection	Initial number	Survivors (at 80 days)	Musculinized female
control	Distilled water	7	2	0
1	Acid-alcohol fraction	7	0	0
2	Acetone precipitated fraction	7	0	0
3	unbound	7	1	0
4	20% acetonitrile	7	0	0
5	40% acetonitrile	7	1	0
6	60% acetonitrile	7	0	0

At 80 days after injection, 2 of 7 postlarvae in control group survived while there was only 1 of 7 survived in the injection of 20% acetonitrile and 60% acetonitrile fraction. However, none of the survivors were successfully reversed to male. The external male characteristics such as appendix muculina, male gonopore, body size and appendages length could not be observed.

RESULTS AND DISSCUSSION

Results

1. Extraction and purification of androgenic gland hormone from freshwater prawn

1.1 Extraction of androgenic gland hormone from freshwater prawn

In order to obtain larger amount of AGH (AGH), hypertrophy of androgenic glands of male freshwater prawn was induced by ablation of one eyestalk. The after 20 days, the gland and vas deferens each was dissected out.
Based on the assumption that AGH was specifically localized in the gland, it must be found only in the gland and not be found in the vas deferens. So, in this experiment both androgenic tissues (androgenic gland plus vas deferens) and vas deferens were extracted and difference in their protein patterns were compared.

The tissues of hypertrophied androgenic tissues and vas deferens were homogenized in acid-alcohol to prevent protease activity and remove large protein molecules. The supernatant was treated twice with cold acetone to precipitate the small proteins or peptides. After solubilization of the precipitate with acetic acid, the extract of each tissue was subjected to reverse phase column chromatography on Sep-Pak C18 cartridge. Protein contents of acetone androgenic tissues and vas deferens extracts were shown in Table 5. Total protein per mg of tissue of the acetone extract of androgenic tissues and vas deferens were nearly the same, 31 and 38 μg per mg tissue, respectively.

Table 5 Protein contents of the acetone androgenic tissues and vas deferens extracts of *Macrobrachium rosenbergii.*

	Androgenic tissues	Vas deferens
Number of prawns	150	150
Total tissue weight (mg)	7.4	13.9
Total protein content (mg)	231.1	531.0
Total protein (μg) per mg tissue	31	38

1.2 Purification of AGH by reverse phase chromatography

An aliquot (25 μg protein) of acetone androgenic tissues and vas deferens extracts each was analyzed by reverse phase HPLC on Sep-Pak C18 cartridge. Protein profile of stepwise eluted by 20%, 40% and 60% acetonitrile containing 0.05% TFA was shown in Fig. 18. The chromatograms obtained from the acetone androgenic tissues (Fig. 18A) and vas deferens extract (Fig. 18B) displayed peaks which were not well resolved. However, fractions of each peak were pooled and analyzed by MALDI-TOF MS. The MALDI-TOF MS spectrum of the acetone extracts, pooled unbound fractions and the acetronitrile fractions of the acetone androgenic tissues and vas deferens extracts were shown in Fig.19-23, respectively.

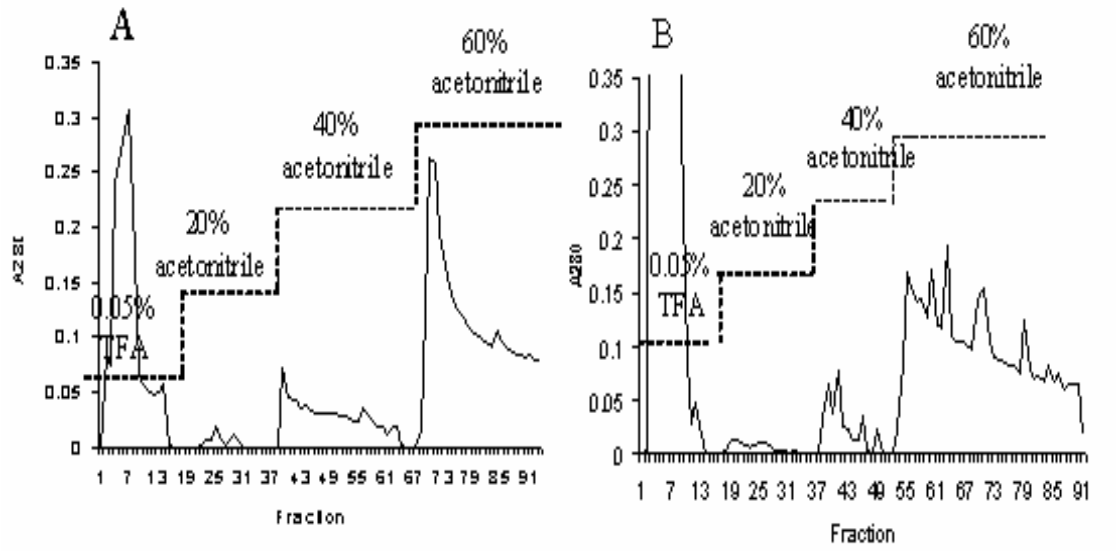


Figure 18 Reverse phase HPLC of acetone extracts of the androgenic tissues and vas deferens on Sep-Pak C_{18} catridge.

The acetone androgenic tissues (A) and vas deferens (B) extracts of the freshwater prawn (25 µg / 5 µl) were applied to a Sep-Pak C_{18} cartridge, pre-equilibrated with 0.05% TFA. The catridge was washed with the equilibration solvent and the adsorbed proteins were eluted stepwise with isocratic 20%, 40% and 60% acetonitrile containing 0.05% TFA at a flow rate of 1 mg/ml. Fractions of 2 ml were collected.

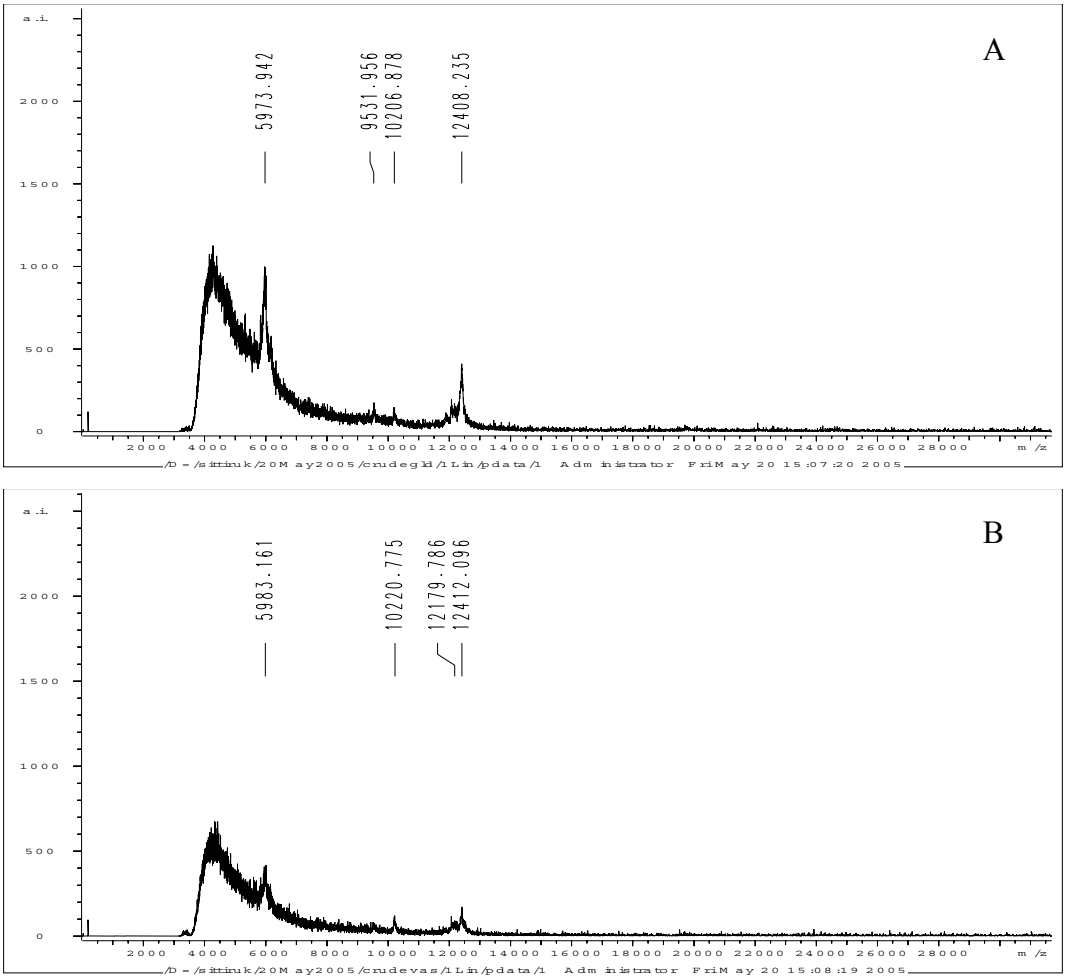


Figure 19 MALDI-TOF MS Spectrum of acetone androgenic tissues (A) and vas deferens (B) extracts

The spectra were recorded in the linear positive ion mode using sinapinic acid matrix. Pooled fractions were collected and evaporated in a vacuum to remove acetonitrile before subject to Bruker Reflex IV matrix assisted laser desorption ionization (MALDI) mass spectrometer equipped with a delayed extraction source and 337 nm pulsed nitrogen laser. One microliter was mixed with 1 µl of 50 mM sinapinic acid and applied to the sample probe. The instrument was run in the linear mode using 20 kV acceleration.

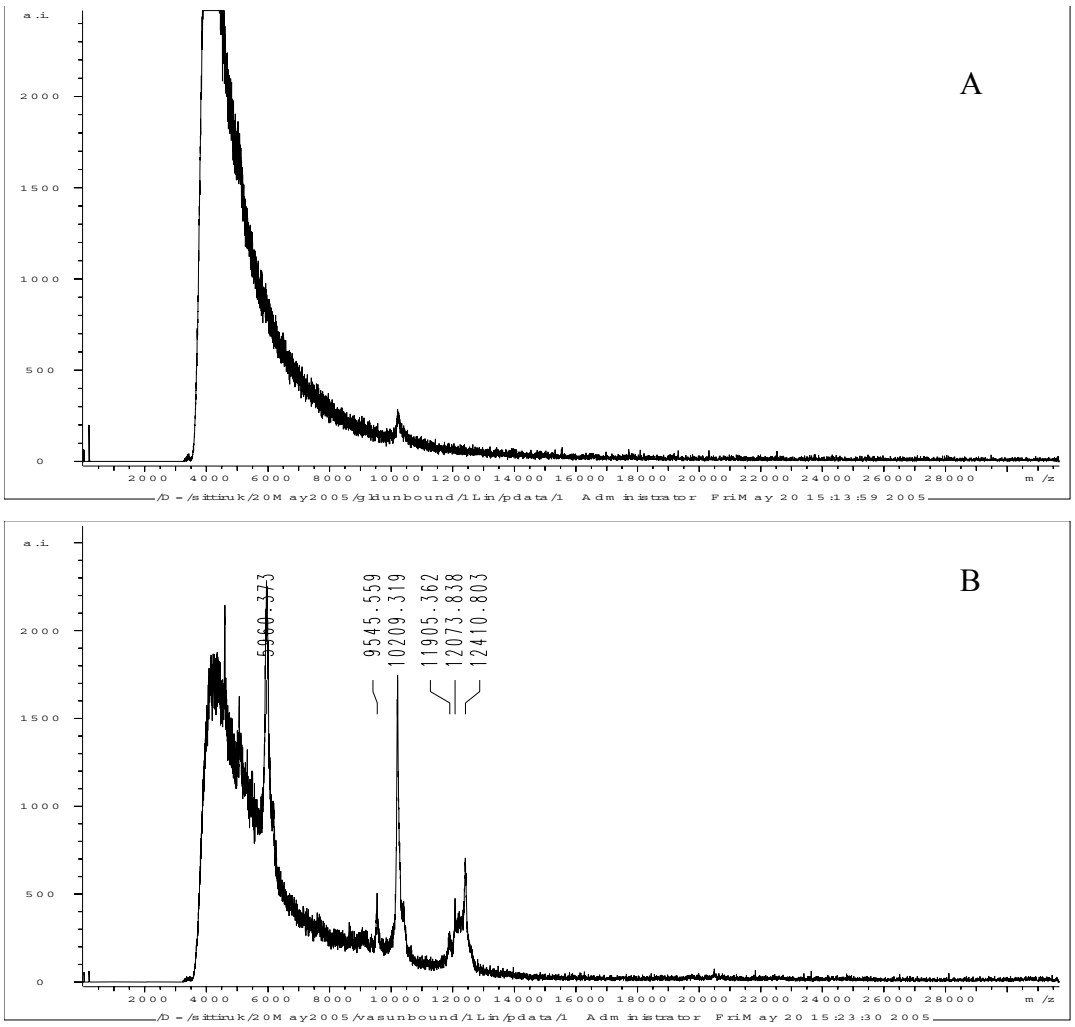


Figure 20 MALDI-TOF MS Spectrum of pooled unbound fractions obtained from RP (Sep-Pak C18 catridge) -HPLC separated acetone androgenic tissues (A) and vas deferens (B) extracts.

The spectra were recorded in the linear positive ion mode using sinapinic acid matrix Pooled fractions were collected and evaporated in a vacuum to remove acetonitrile before subject to Bruker Reflex IV matrix assisted laser desorption ionization (MALDI) mass spectrometer equipped with a delayed extraction source and 337 nm pulsed nitrogen laser. One microliter was mixed with 1 µl of 50 mM sinapinic acid and applied to the sample probe. The instrument was run in the linear mode using 20 kV acceleration.

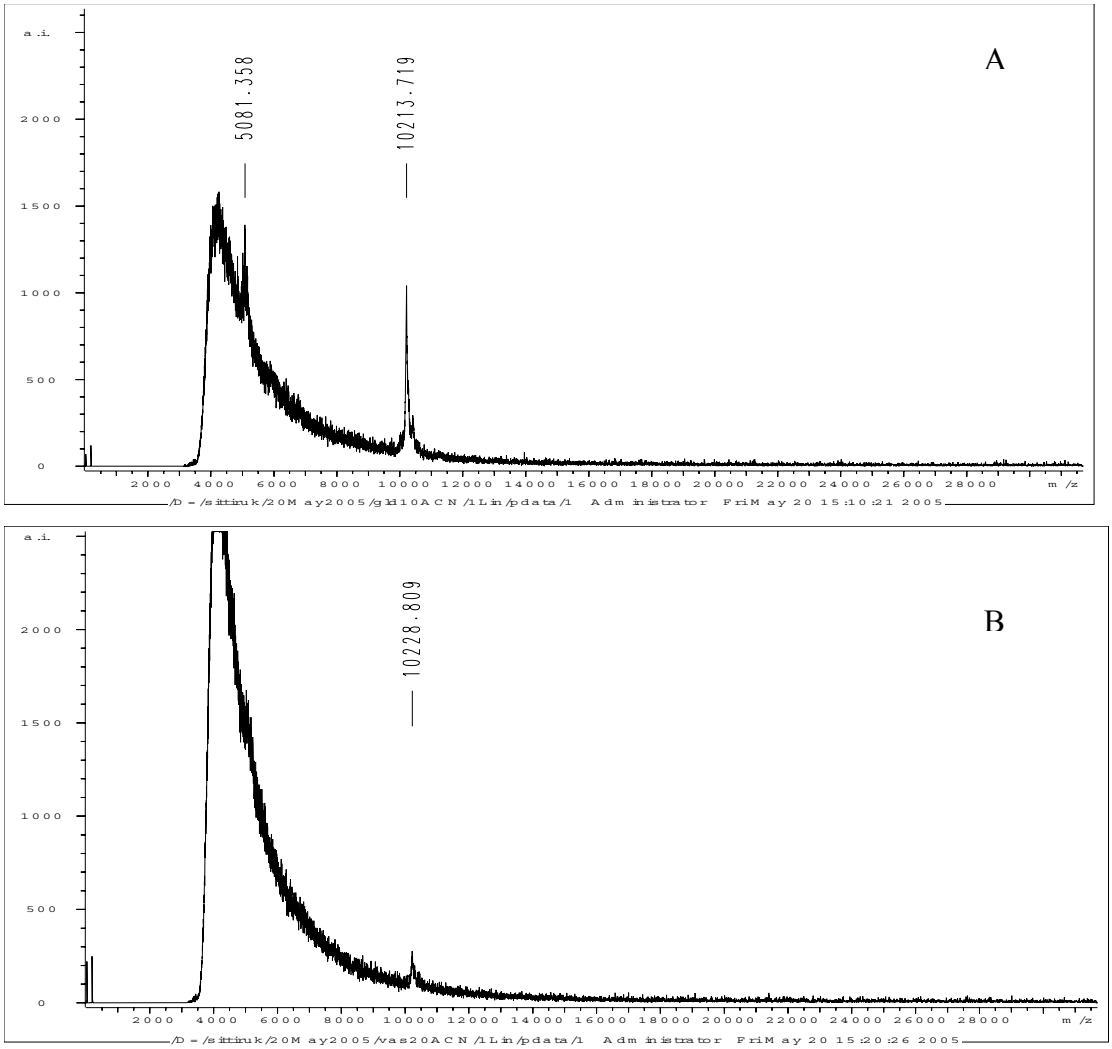


Figure 21 MALDI-TOF MS Spectrum of pooled 20% acetronile fractions obtained from RP(Sep-Pak C18 catridge)-HPLC separated acetone androgenic tissues (A) and vas deferens (B) extracts.

The spectra were recorded in the linear positive ion mode using sinapinic acid matrix. Pooled fractions were collected and evaporated in a vacuum to remove acetonitrile before subject to Bruker Reflex IV matrix assisted laser desorption ionization (MALDI) mass spectrometer equipped with a delayed extraction source and 337 nm pulsed nitrogen laser. One microliter was mixed with 1 µl of 50 mM sinapinic acid and applied to the sample probe. The instrument was run in the linear mode using 20 kV acceleration.

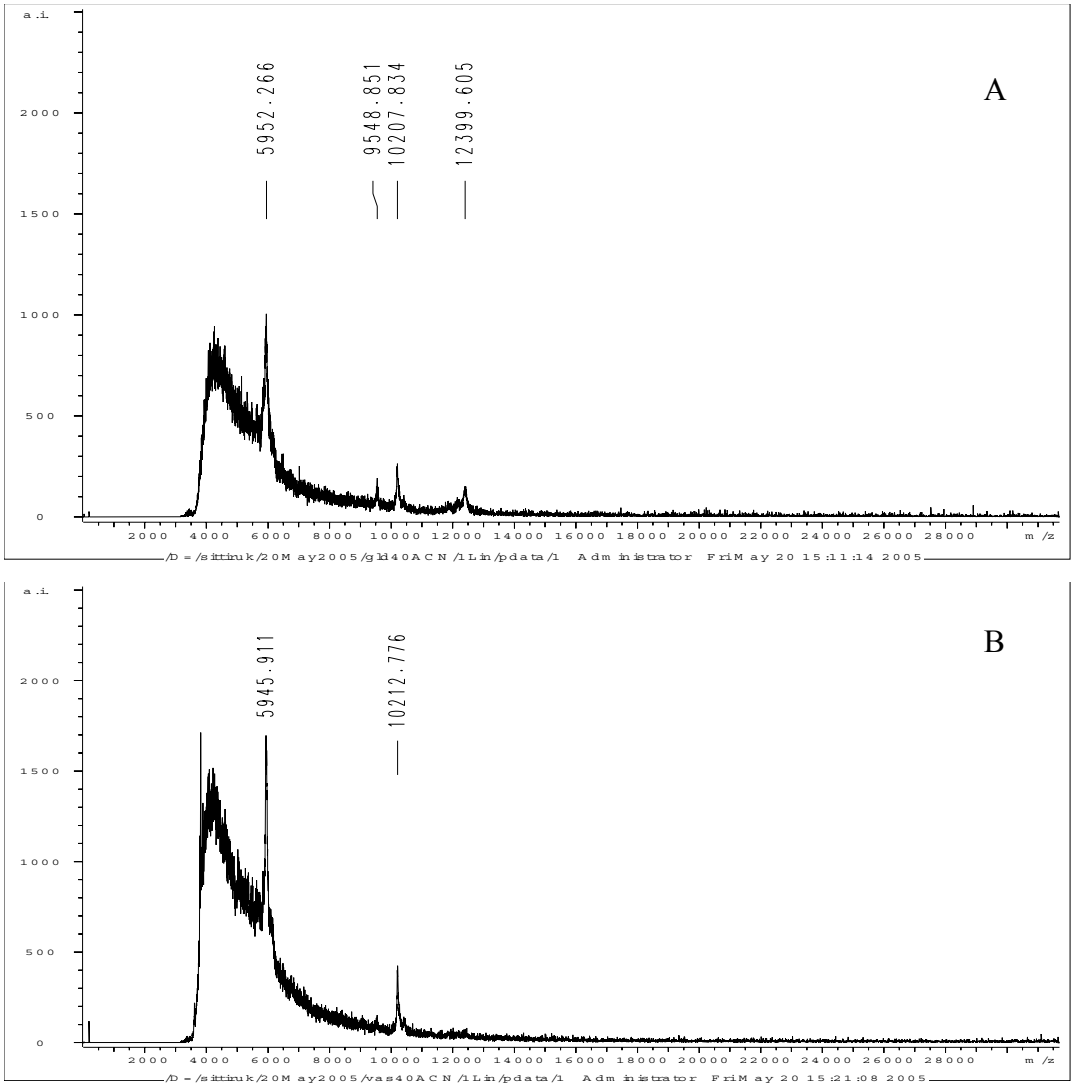


Figure 22 MALDI-TOF MS Spectrum of pooled 40% acetronile fractions obtained from RP(Sep-Pak C18 catridge)-HPLC separated acetone androgenic tissues (A) and vas deferens (B) extracts.

The spectra were recorded in the linear positive ion mode using sinapinic acid matrix. Pooled fractions were collected and evaporated in a vacuum to remove acetonitrile before subject to Bruker Reflex IV matrix assisted laser desorption ionization (MALDI) mass spectrometer equipped with a delayed extraction source and 337 nm pulsed nitrogen laser. One microliter was mixed with 1 μl of 50 mM

sinapinic acid and applied to the sample probe. The instrument was run in the linear mode using 20 kV acceleration.

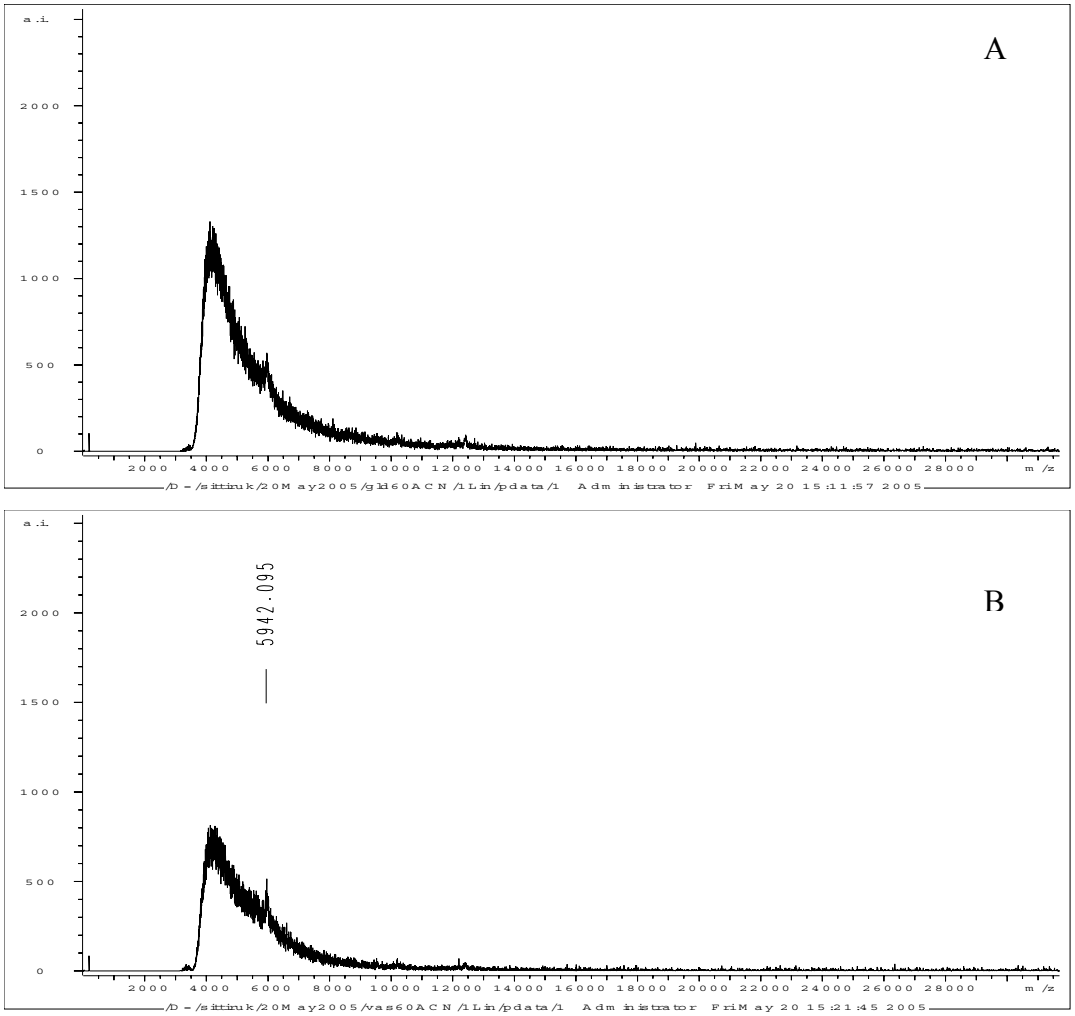


Figure 23 MALDI-TOF MS Spectrum of pooled 60% acetronile fractions obtained from RP(Sep-Pak C18 catridge)-HPLC separated acetone androgenic tissues (A) and vas deferens (B) extracts.

The spectra were recorded in the linear positive ion mode using sinapinic acid matrix. Pooled fractions were collected and evaporated in a vacuum to remove acetonitrile before subject to Bruker Reflex IV matrix assisted laser desorption ionization (MALDI) mass spectrometer equipped with a delayed extraction source and 337 nm pulsed nitrogen laser. One microliter was mixed with 1 µl of 50 mM

sinapinic acid and applied to the sample probe. The instrument was run in the linear mode using 20 kV acceleration.

No product was observed in the RP-HPLC unbound fractions of androgenic tissues (Fig. 19A) which differed from the vas deferens fractions (Fig. 19B) whose molecular masses were read in the range 5.9 – 12.4 kDa. The result implies the different of protein composition in the acetone androgenic tissues and vas deferens extracts. Peak of molecular mass approximately 10.2 kDa was found in 20% and 40% acetronitrile fractions of both the acetone androgenic tissues and vas deferens extracts. However, much more different in the peak height of acetone androgenic tissues extract when compared to those of the vas deferens extract was shown in the 20% acetronitrile fractions.

The acetone angrogenic tissue and vas deferens extracts were re-chromatrographied by reverse phase HPLC, on a Lichrosorb C18 column and the bound products were eluted with a linear gradient of 0-100 % acetonitrile in 0.05% TFA. The RP-HPLC chromatogram displayed complex peaks which were not well resolved as shown in Fig. 24. Each fraction was analyzed by MALDI-TOF MS and it was found that at 42.5% acetronitrile, the fraction no. 22 of the acetone AG extract contained a major protein of molecular mass 10.2 kDa (Fig 25A) while 10.2 kDa spectrum was not found in the fraction no. 22 of the acetone vas deferens extract (Fig 25B). This 10.2 kDa peak is assumed to be the AGH because it was presented only in the androgenic tissues.

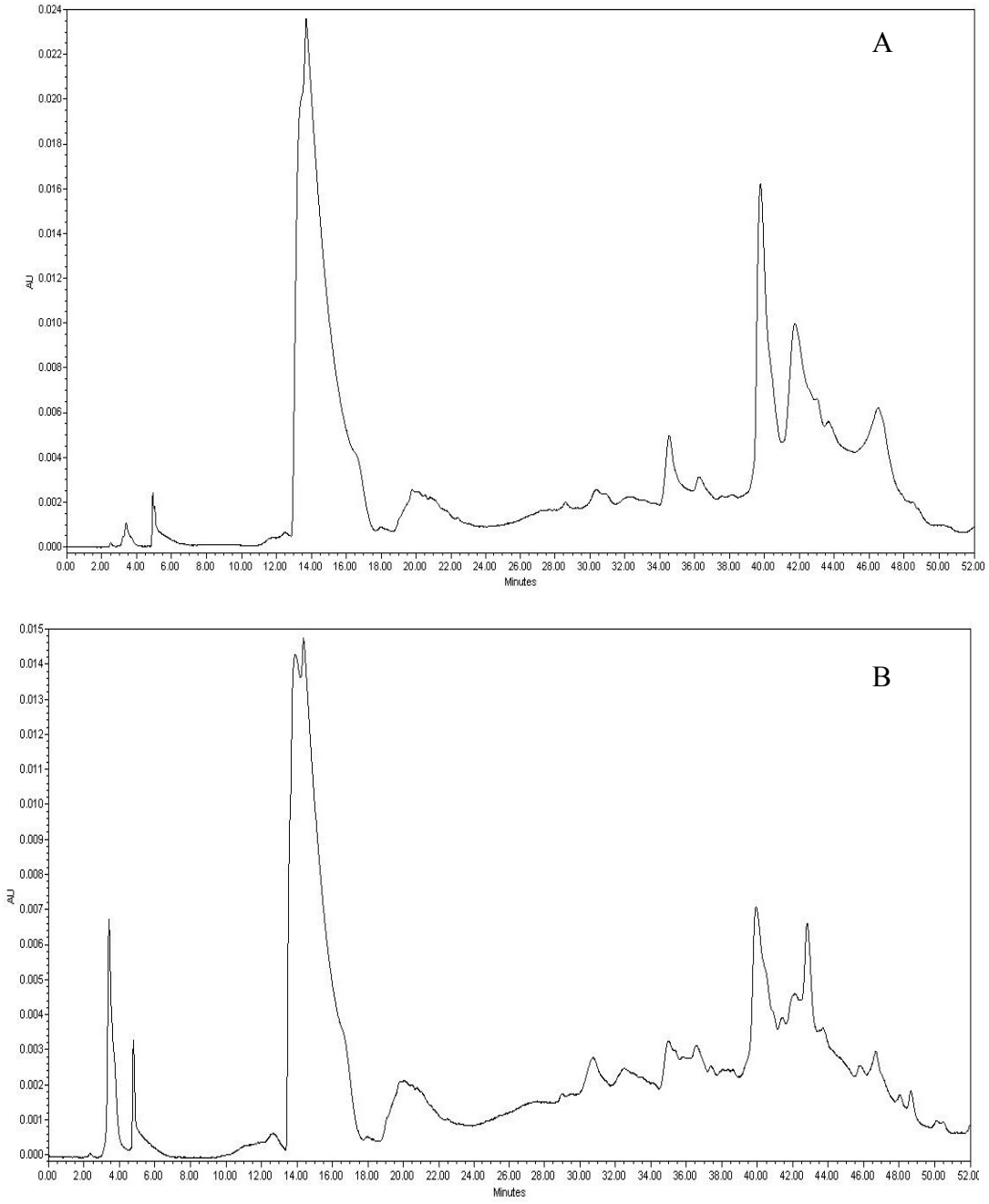


Figure 24 Reverse phase HPLC of acetone extracts of the androgenic tissues (A) and vas deferens (B) on Lichrosorb column.

The acetone androgenic tissues (A) and vas deferens (B) extracts of the giant freshwater prawn (25 µg / 5 µl) were applied to a Lichrosorb column, pre-equilibrated with 0.1% TFA. The column was washed with the equilibration solvent

and the adsorbed materials were gradient eluted with 0-100 % acetonitrile containing 0.1% TFA at a flow rate of 1 mg/ml. Fractions of 1 ml were collected.

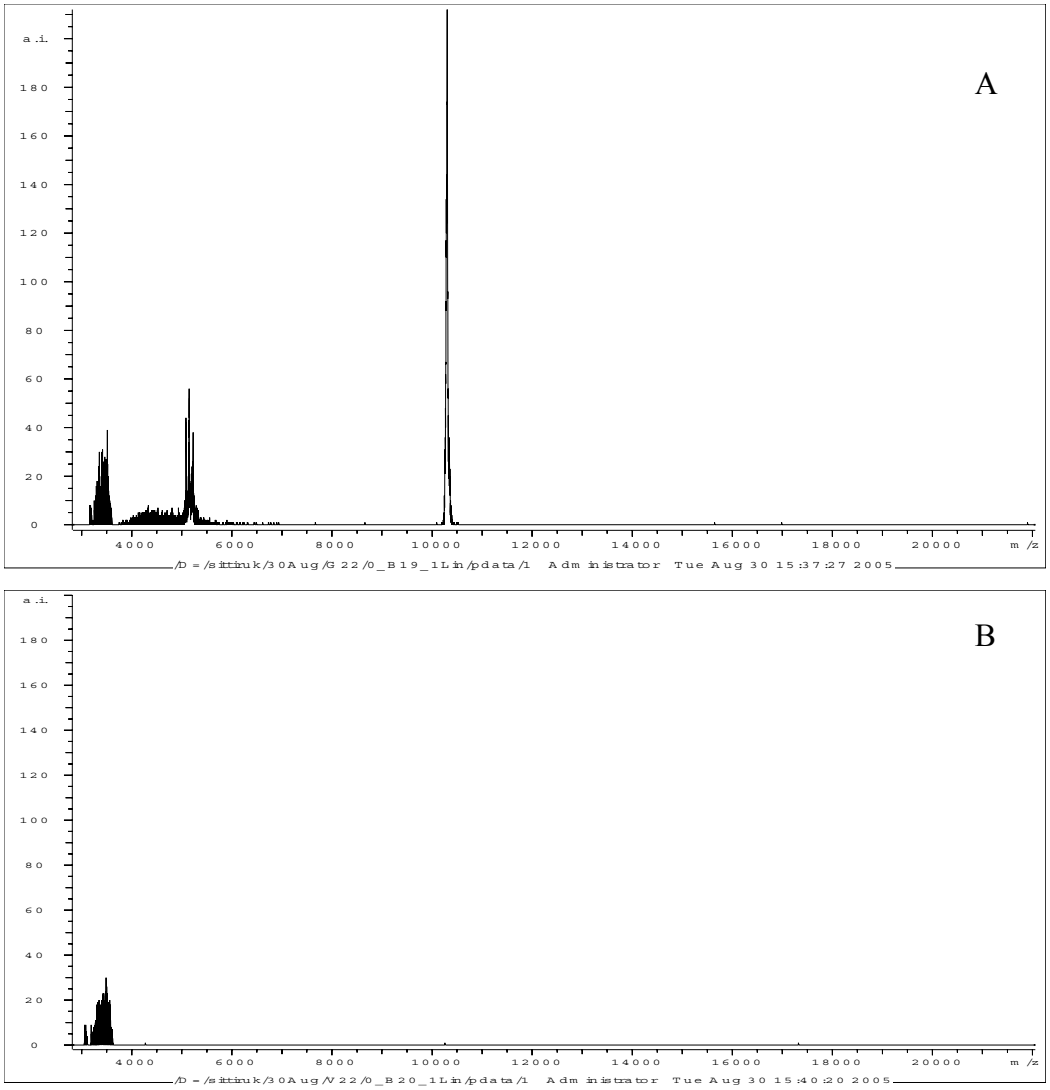


Figure 25 MALDI-TOF MS Spectrum of acetronile fraction no. 22 obtained From RP (Lichrosorb)-HPLC separated acetone androgenic tissues (A) and vas deferens (B) extracts.

The spectra were recorded in the linear positive ion mode using sinapinic acid matrix. Pooled fractions were collected and evaporated in a vacuum to remove acetonitrile before subject to Bruker Reflex IV matrix assisted laser desorption ionization (MALDI) mass spectrometer equipped with a delayed extraction source

and 337 nm pulsed nitrogen laser. One microliter was mixed with 1 μl of 50 mM sinapinic acid and applied to the sample probe. The instrument was run in the linear mode using 20 kV acceleration.

1.3 Characterization of AGH structure

Because there was some reports of androgenic hormone in other crustaceans having one N-glycosylation and two polypeptides which were linked by two disulfide brigdes (Martin *et al.*, 1999; Ohira *et al.*, 2003). In this experiment, the 10.2 kDa RP-HPLC purified protein of androgenic tissues was investigated for its structure using enzymatic deglycosylation by N-Glycosidase F (PNGase F) and reduction of disulfide bridges by dithiothrietol (DTT). Then, the reaction mixtures were analyzed by MALDI-TOF MS. The results obtained in this study did not show the different mass when compared to the mass before deglycosylation and reduction (Figure 26). This implied that the glycan moiety and disulfide bridges between 2 peptide chains were not present on the 10.2 kDa protein.

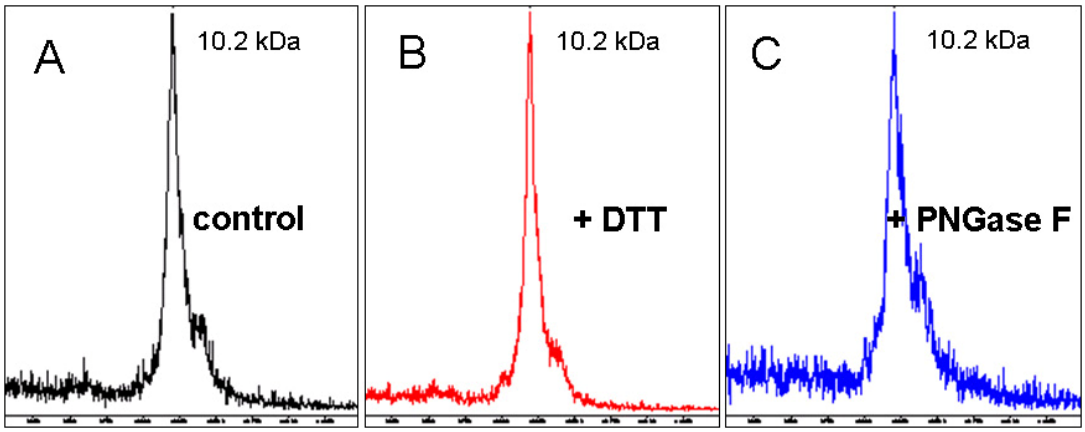


Figure 26 MALDI-TOF MS Spectra of the RP-HPLC 10.2 kDa protein purified from androgenic tissues (A), after reduction of disulfide bridges with DTT (B) and enzymatic deglycosylation with PNGase F (C).

The instrument Bruker Reflex IV MALDI was run in the linear mode using 20 kV acceleration. The 1 µl of control or deglycosylated or reduced proteins were mixed with 1 µl of matrix solution (20 mg/ml of sinapinic acid in acetonitrile/0.1% TFA, 50:50, v/v). Finally, 0.5 µl of the mixture was deposited onto the MALDI as target plate. All spectra are the results of signal averaging of 200 shots.

1.4 Identification of AGH by peptide mass fingerprint using endoproteinase Lys-C digestion

In order to characterize the molecular basis of the RP-HPLC purified 10.2 kDa protein, it was subjected to identify by peptde mass fingerprint. A combination of endoproteinase Lys-C digestion and in silico matching of peptides masses against protein databases was used. The MALDI-TOF MS spectrum of endoproteinase Lys-C digested peptides was shown in Fig. 27. The peptide mass fingerprint analysis by MASCOT search did not show the results matching to AGHs of any crustaceans available in the NCBI database (data not shown).

1.5 Protein analysis by tricine-sodium dodecyl sulfate polyacrylamide gel Electrophoresis

The acetone extracts of androgenic tissues and vas deferens were analyzed for the different in their protein band patterns using 15% tricine-SDS polyacrylamide gel electrophoresis (tricine-SDS-PAGE). Several polypeptide bands at 9.7, 10.7, 12.5 and 13.6 kDa, were detected in both tissues by coomassie blue staining as shown in Fig. 28. These polypeptides were selected to be analyzed by LC/MS. Moreover, the another two protein bands at about 10 and 15 kDa which were seen in the other gel running (data not shown) were also selected to be analyzed.

1.6 *De novo* sequencing of androgenic gland hormone by LC/MS

The 9.7, 10.7, 12.5, and 13.6 kDa proteins from tricine-SDS-PAGE each was in-gel digested by trypsin and the amino acid sequence of tryptic peptides was determined by LC-MS-MS. The internal peptide sequences derived from trypsin digestion were shown in Table 6. All of the possible sequences were used to do similarity search against the amino sequence database in the NCBI, using BlastP program. There was no match with any proteins in the database. Nevertheless, these sequences were used to design some degenerated primers specific to the AGH as shown in Table 3.

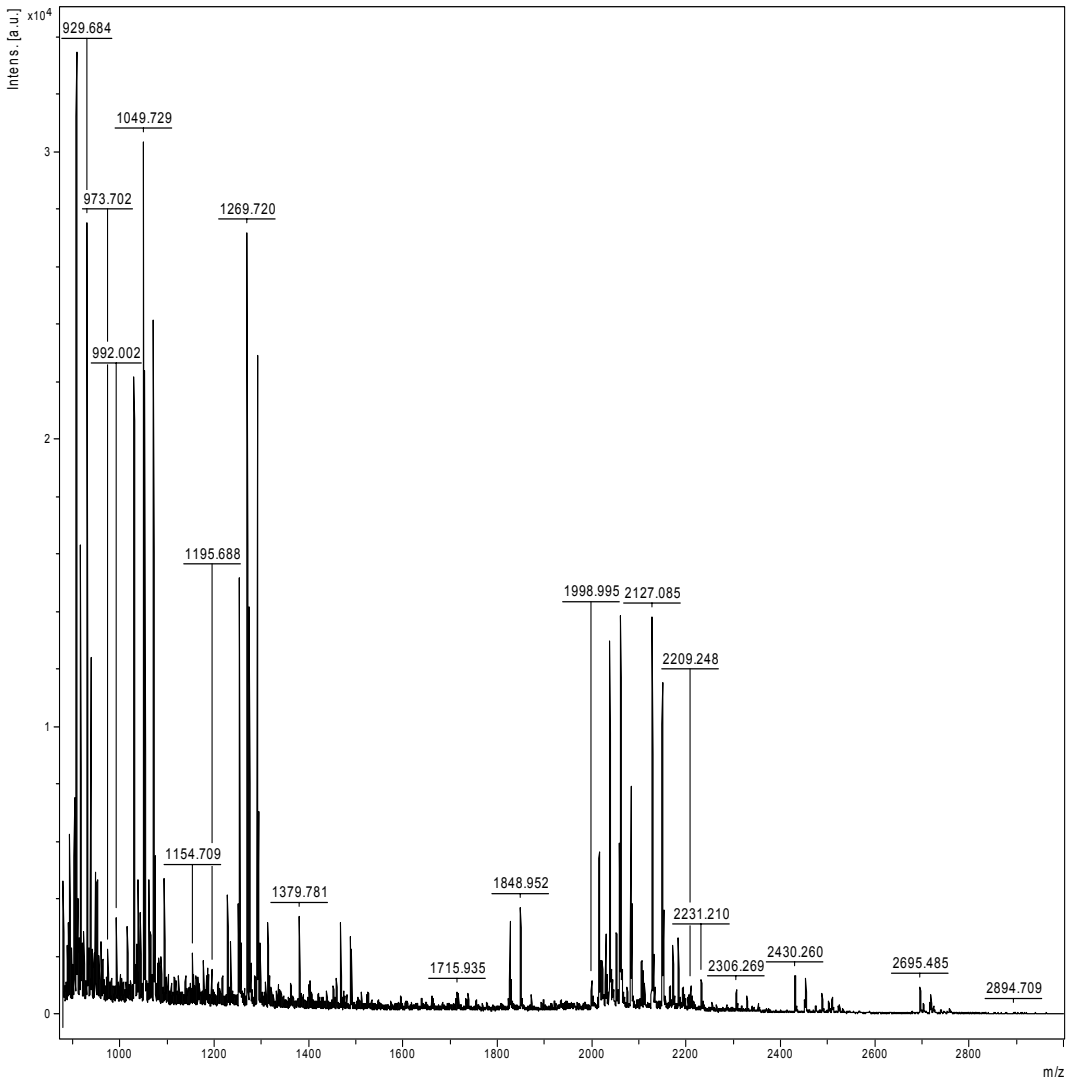


Figure 27 The MALDI-TOF MS Spectrum of peptides from endoproteinase Lys-C digested of RP-HPLC purified 10.2 kDa protein from androgenic tissues.

The digested peptide mixture was mixed with saturated 2,5-dihydroxybenzoic acid (DHBA) matrix solution and spotted onto the MALDI target plate. The instrument Bruker Reflex IV MALDI was run in the refractor mode using 20 kV acceleration.

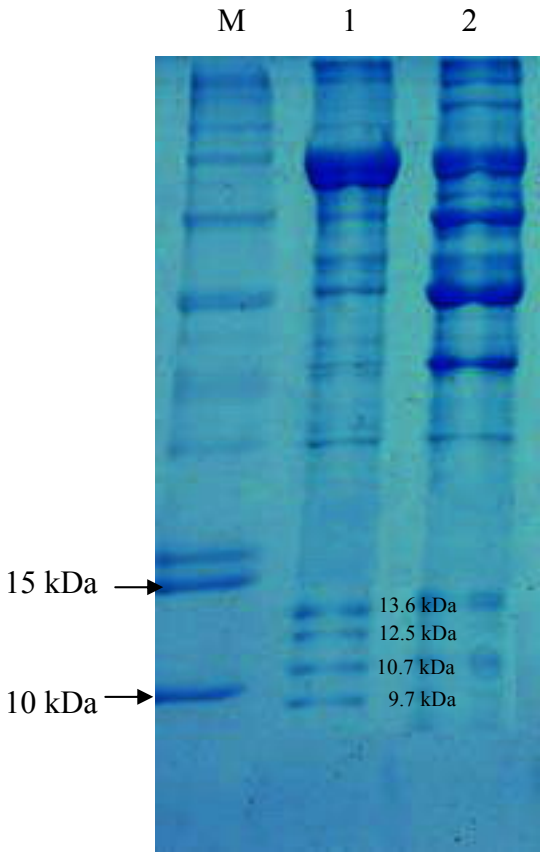


Figure 28 Tricine-SDS-PAGE of acetone extracts of androgenic tissues and vas deferens from freshwater prawn using 15% gel.
Lane1, marker proteins; Lane 2, acetone extract of androgenic tissues (30 µg); Lane 3, acetone extract of vas deferens.

Table 6 Sequences of the peptide fragments derived from tryptic digested peptides of *Machrobrachiun rosenbergii* androgenic tissues analyzed by LC-MS-MS

Protein Size	Peptide MW (Da)	Number of Amino acid	Sequence	Retention Time (min)
15	1282.667	11	(-)ELGAVLDWQPR(-)	27.91
	1282.667	11	(-)LEGAVLDWQPR(-)	27.91
	1395.663	15	(-)SAQSAYEALGAGSGK(-)	24.79
	1395.663	15	(-)ASQSAYEALGAGSGK(-)	24.79
	1395.663	15	(-)TGQSAYEALGAGSGK(-)	24.79
	1395.663	15	(-)SAQSAYEAGLAGSGK(-)	24.79
	1395.663	15	(-)ASQSAYEAGLAGSGK(-)	24.79
	1394.679	15	(-)SAQSAYQALGAGSGK(-)	24.79
	1408.695	16	(-)GVGSSAYQAGLAGSGK(-)	25.78
	1408.695	16	(-)VGGSSAYQAGLAGSGK(-)	25.78
	1408.706	15	(-)RGSSAYQAGLAGSGK(-)	25.78
	1408.695	15	(-)GVGSSAYQQLAGSGK(-)	25.78
	1408.695	16	(-)GVSGSAYQAGLAGSGK(-)	25.78
	1402.721	15	(-)GLSGAAYQGQLGPGK(-)	27.85
	1402.721	15	(-)GLGSAAYQGQLGPGK(-)	27.85
15	1402.721	16	(-)GLSGAAYQGGLAGPGK(-)	27.85
	1402.721	15	(-)AVSGAAYQGQLGPGK(-)	27.85
	1402.721	15	(-)LGGSAAYQGQLGPGK(-)	27.85
	1268.673	13	(-)ELGAVLTGAEGPR(-)	27.91
	1268.673	13	(-)ELGAVLTGEAGPR(-)	27.91
	1268.673	13	(-)LEGAVLTGEAGPR(-)	27.91
	1268.673	12	(-)ELGAVLTQEGPR(-)	27.91
	1268.673	13	(-)ELGAVLTGEAPGR(-)	27.91
	1268.672	13	(-)ELGAVLTGGPEAR(-)	29.09
	1268.672	13	(-)LEGAVLTGGPEAR(-)	29.09
	1268.677	13	(-)ELGAVLTGGPLWG(-)	29.09

Table 6 (Continue)

Protein Size	Peptide MW (Da)	Number of Amino acid	Sequence	Retention Time (min)
10	1065.546	9	(-)VFDETTARK(-)	25.21
	1065.534	9	(-)VFDETTNLK(-)	25.21
	1065.534	9	(-)FVDETTNLK(-)	25.21
	1065.534	10	(-)VFDETTGGLK(-)	25.21
	1065.534	9	(-)VFDETTNKL(-)	25.21
	806.4075	6	(-)LAWDFR(-)	25.3
	1032.524	8	(-)TLLEWESR(-)	25.35
	1032.524	8	(-)LTLEWESR(-)	25.35
	1025.518	8	(-)VSEYLYPR(-)	25.75
	806.4075	6	(-)ALWDFR(-)	25.3
9.7	1226.6367	11	(-)PGSLSNLQQQR(-)	26.58
10.7	807.3763	11	(K)QFDAAEK(A)	18.97
12.5	1407.6517	12	(-)DEDLASEVLDFR(-)	29.98
13.6	887.46	7	(-)ELLEQEK(-)	23.05
	887.46	7	(-)LELEQEK(-)	23.05
	847.4803	8	(-)LGGYPTLK(-)	22.37

2. Molecular cloning of androgenic gland hormone from cDNA

2.1 Cloning and sequencing of the partial cDNA fragments encoding androgenic gland hormone

Total RNA was extracted from androgenic glands from 12 male prawns which has average weight about 100 g per prawn using TRIzol reagent and was used as a template for RT-PCR. Total weight of extracted gland is 211 mg. The isolated total RNA had an A 260/A280 ratio of 1.83 suggesting that the RNA had high quality without any degraded RNA and contaminated genomic DNA. Yield of the total RNA was approximately 0.42 μg per mg tissues. Total RNA was run on 1% agarose gel and the two expected bands of 18s and 28s rRNA were observed as shown in Fig. 29

All PCR products derived from pairing AGHm-F1, AGHm-F2, AGHm-F3, AGHm-R1, AGHm-R2, AGHm-R3 and AGHm-R4 primers (synthesized based on the nucleotide sequence of the AGH from isopods) have the lower size than the expected size. PCR amplification derived from pairing between AGHm-F2/AGHm-R2, AGHm-F3/AGHm-R2, AGHm-F2 /AGHm-R3 and AGHm-F3/ AGHm-R3 gave many bands (Fig. 30A: lane 6, 7, 9 and 10, respectively) which were non-specific products. As same as PCR amplification using AGHm-F4, AGHm-F5, AGHm-R5 and AGHm-R6 primers (synthesized based on the amino acid sequences of *Cherex quadricarinatus* insulin-like androgenic gland factor), there were many non-specific PCR products too. However, PCR products derived from pairing between AGHm-F2/ AGHm-R2 and AGHm-F5 /AGHm-R6 (Fig. 30A: lane 2 and Fig. 30B: lane 5, respectively) gave an interesting band with the nearly expected size. However, The PCR products of the expected size and the PCR products which gave the sharp band were selected to clone into pGEM-T easy and transformed into *E. coli* JM 109 and the nucleotide sequence of each clones was determined.

The nucleotide sequences of the PCR products were compared to AGH from *A. vulgare*, *P. scaber*, *P. dillatatus* and of *C. quadricarinatus* insulin-like androgenic gland factor. There was no similarity among them significantly (data not

shown). However, the deduced amino acid sequences of these products were further analysed.

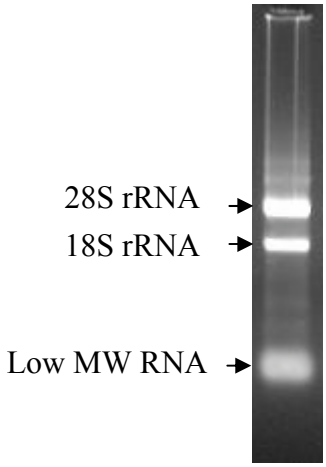
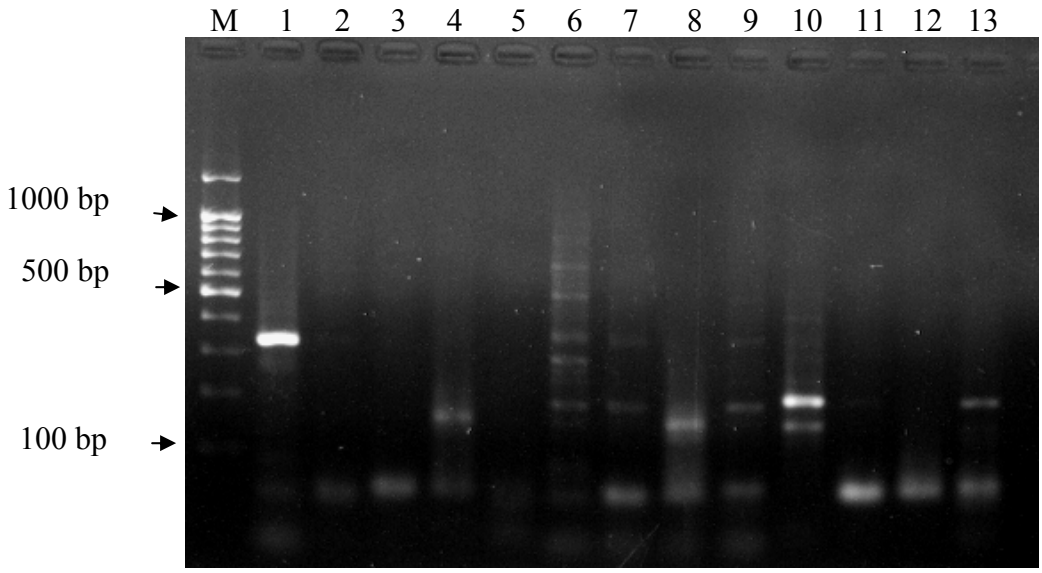


Figure 29 Agarose gel electrophoresis of total RNA from androgenic glands of *M. rosenbergii* for molecular cloning of androgenic gland hormone.

Total RNA was separated by electrophoresed on 1% agarose gel with MOPS buffer at 100 volts for 20 min, stained with ethidium bromide and visualized under UV transilluminator.

Figure 30 (A) PCR fragments amplified by primers based on the nucleotide sequence of the androgenic gland hormone from *Armadillidium vulgare*, *Porcellio scaber* and *P. dillatatus* of 26S rRNA 250 bp. (lane 1: actin as a control (expected sized 337 bp),lane 2: AGHm-F1 and AGHm-R1 (expected sized 110 bp), lane 3: AGHm-F2 and AGHm-R1 (expected sized 110 bp), lane 4: AGHm-F3 and AGHm-R1 (expected sized 50 bp), lane 5: AGHm-F1 and AGHm-R2 (expected sized 130 bp), lane 6: AGHm-F2 and AGHm-R2 (expected sized 130 bp), lane 7: AGHm-F3 and AGHm-R2 (expected sized 70 bp), lane 8: AGHm-F1 and AGHm-R3 (expected sized 299 bp), lane 9: AGHm-F2 and AGHm-R3 (expected sized 299 bp), lane 10: AGHm-F3 and AGHm-R3 (expected sized 239 bp), lane 11: AGHm-F1 and AGHm-R4 (expected sized 327 bp), lane 12: AGHm-F2 and AGHm-R4 (expected sized 327 bp), lane 13: AGHm-F3 and AGHm-R4 (expected sized 267 bp), Lane M: 100 bp ladder plus 1.5 kb)

(B) PCR fragments amplified by primers based on the nucleotide and amino acid sequences of *Cherex quadricarinatus* insulin-like androgenic gland factor. (lane 1: actin as a control (expected sized 337 bp), lane 2: AGHm-F4 and AGHm-R5 (expected sized 349 bp), lane 3: AGHm-F5 and AGHm-R5 (expected sized 292 bp), lane 4: AGHm-F4 and AGHm-R6 (expected sized 421 bp), lane 5: AGHm-F5 and AGHm-R6 (expected sized 364 bp), lane M: 100 bp ladder plus 1.5 kb)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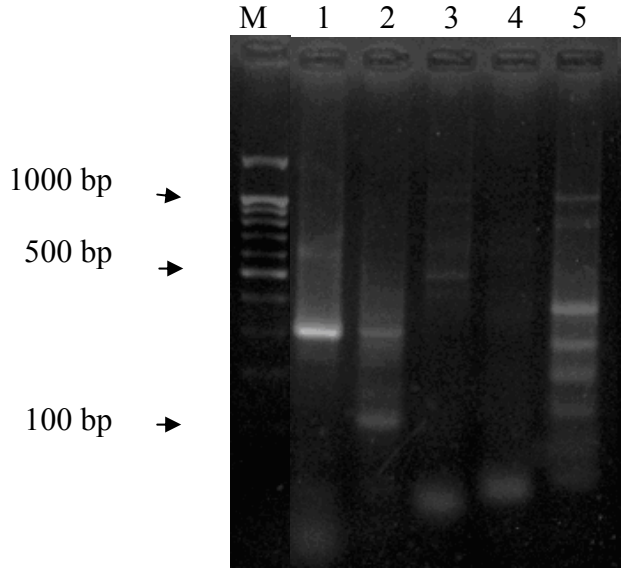
500 bp
100 bp

A

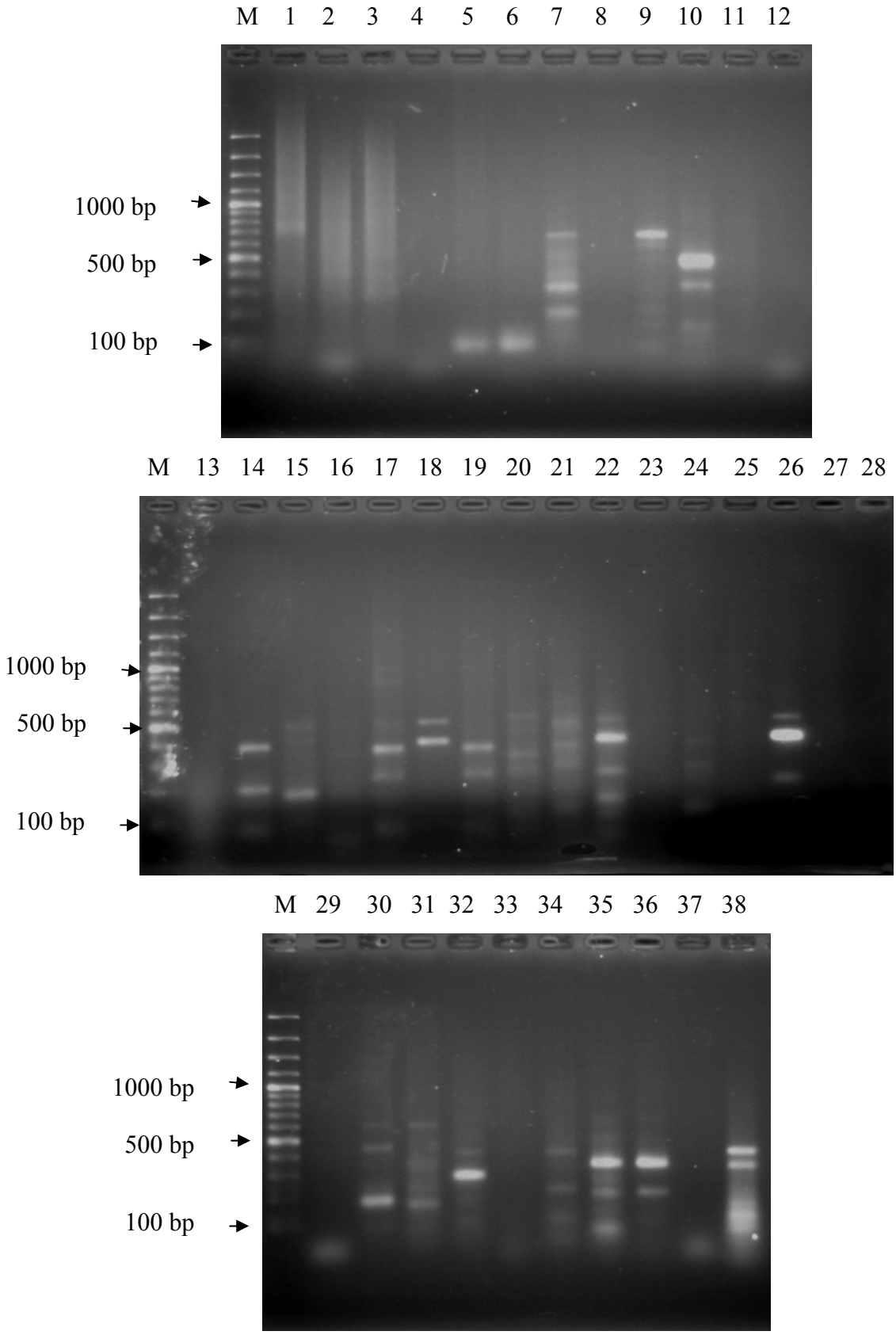

M 1 2 3 4 5
1000 bp
500 bp
100 bp

B

The result of the amplification using the degenerated primer designed from amino acid sequences of 9.7, 10.7, 12.5, 13.6, 10, and 15 kDa peptide fragments (10D_F1, 10D_F2, 10D_F3, 10D_F4, 10D_RF1, 10D_R2, 10D_R3, 10D_R4, 15D2_F1, 15D2_F2, 15D2_F3, 15D2_R1, 15D2_R2, 15D2_R3, AG9.7_F1, AG10.7_F1, AG12.5_F1, AG13.8_F1, AG13.8_F2, AG13.8_F3, AG13.8_R1, AG13.8_R2 and AG13.8_R3) was shown in the Fig. 31. PCR procedure was as described above. There were many non-specific PCR products. Some pairs of primers gave more than one band and some did not give any band. All of PCR products derived from AG13.8_F2/AG13.8_R3, AG13.8_F3 /AG13.8_R1, 10D_F1/10D_R2, 10D_F2/10D_R1, 10D_F2/10D_R2, 10D_F3/10D_R2, 15D2_F2/15D2_R1, 15D2_F3/15D2_R1 and 15D2_F3/15D2_ R2 (lane 9, 10, 14, 17, 18, 22, 26, 32, 35 and 36) which gave sharp and intense band were selected to clone into pCR 8 and transform into *E. coli* Mach1 -T1 and the nucleotide sequence of each clones was determined.

The nucleotide sequences of the PCR products were compared to AGH from *A. vulgare*, *P. scaber*, *P. dillatatus* and of *C. quadricarinatus* insulin-like androgenic gland factor. There was no similarity among them significantly (data not shown). the The nucleotide sequences of these products were further analysed by BlastX. However, they did not share significant sequence homology with any known sequence in the databases.

Figure 31 PCR fragments amplified by primers based on the degenerated primers designed from amino acid sequences of 9.7, 10.7, 12.5, 13.6, 10, and 15 kDa peptide fragments. (Lane 1: AG9.7_F1 and oligo d(T), Lane 2: AG10.7_F1 and oligo d(T),Lane 3: AG12.5_F1 and oligo d(T) Lane 4: AG13.8_F1 and AG13.8_R1, Lane 5: AG13.8_F1 and AG13.8_R2, Lane 6: AG13.8_F1 and AG13.8_R3, Lane 7: AG13.8_F2 and AG13.8_R1, Lane 8: AG13.8_F2 and AG13.8_R2, Lane 9: AG13.8_F2 and AG13.8_R3, Lane 10: AG13.8_F3 and AG13.8_R1, Lane 11: AG13.8_F3 and AG13.8_R2, Lane 12: AG13.8_F3 and AG13.8_R3, Lane 13: 10D_F1 and 10D_R1, Lane 14: 10D_F1 and 10D_R2, Lane 15: 10D_F1 and 10D_R3, Lane 16: 10D_F1 and 10D_R4, Lane 17: 10D_F2 and 10D_R1, Lane 18: 10D_F2 and 10D_R2, Lane 19: 10D_F2 and 10D_R3, Lane 20: 10D_F2 and 10D_R4, Lane 21: 10D_F3 and 10D_R1, Lane 22: 10D_F3 and 10D_R2, Lane 23: 10D_F3 and 10D_R3, Lane 24: 10D_F3 and 10D_R4, Lane 25: 10D_F4 and 10D_R1, Lane 26: 10D_F4 and 10D_R2, Lane 27: 10D_F4 and 10D_R3, Lane 28: 10D_F4 and 10D_R4, Lane 29: 15D2_F1 and 15D2_R1, Lane 30: 15D2_F1 and 15D2_R2, Lane 31: 15D2_F1 and 15D2_R3, Lane 32: 15D2_F2 and 15D2_R1, Lane 33: 15D2_F2 and 15D2_R2, Lane 34: 15D2_F2 and 15D2_R3, Lane 35: 15D2_F3 and 15D2_R1, Lane 36: 15D2_F3 and 15D2_R2, Lane 37: 15D2_F3 and 15D2_R3, Lane 38: actin as control)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100 bp
M 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
1000 bp
500 bp
100 bp
M 29 30 31 32 33 34 35 36 37 38
1000 bp
500 bp
100 bp

2.2 Androgenic gland cDNA library construction

Total RNA was extracted from androgenic glands from 9 prawns which had average weight about 100 g per prawn. The total weight of 18 glands was 180 mg. The isolated total RNA had an A 260/A280 ratio of 2.17 suggesting that the RNA had high quality. Total RNA was 38 µg and yield of the RNA was approximately 0.42 µg total RNA per mg of tissues. Total RNA was run on 1% agarose gel and the two expected bands of 18s and 28s rRNA were observed (Fig. 32)

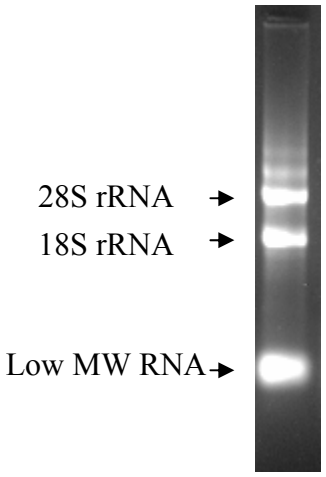

Figure 32 Agarose gel electrophoresis of total RNA from androgenic glands of *M. rosenbergii* for androgenic gland hormone cDNA library construction. Total RNA was separated by electrophoresed on 1% agarose gel with MOPS buffer at 100 volts for 20 min, stained with ethidium bromide and visualized under UV transilluminator.

Messenger RNA was purified from the total RNA of androgenic of *M. rosenbergii* by NucleoTrap mRNA Purification Kits (MACHEREY-NAGEL). The 98.4 ng of mRNA was obtained from 38 µg total RNA. Messenger RNA isolated from total RNA of giant freshwater prawn androgenic gland was subjected for cDNA synthesis and cDNA library construction. Messenger RNA run on 1% agarose gel had good quality with no degradation (Fig. 33)

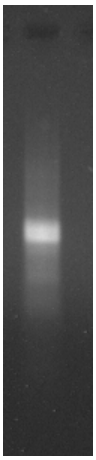

Figure 33 Agarose gel electrophoresis of messenger RNA from androgenic glands of *M. rosenbergii* for androgenic gland hormone cDNA library construction. Messenger Total RNA was separated by electrophoresed on 1% agarose gel with MOPS buffer at 100 volts for 20 min, stained with ethidium bromide and visualized under UV transilluminator.

Fifty micrograms of messenger RNA was then used as a starting material template for *M. rosenbergii* AGH cDNA library construction. The second-strand cDNA was separated by CHROMA SPIN-400 column to fractionate the appropriate cDNA size for further library construction. (Fig. 34) The first four fractions containing cDNA (fractions number 6, 7, 8 and 9) were collected and pooled. The fractionated full-length cDNAs were directionally cloned into TriplEx2 phagemid and transducted into *E. coli* XL1-B.

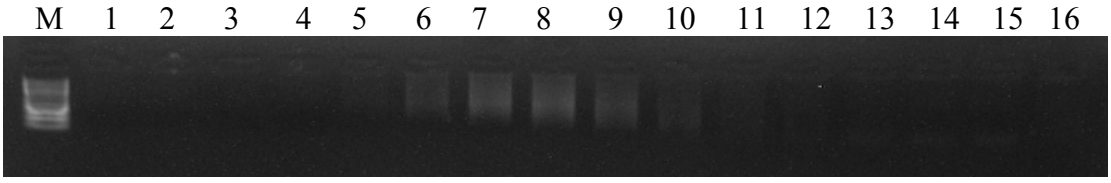

Figure 34 Double-strand cDNA fractionated by CHROMA SPIN-400 column separated by electrophoresed on 1.1% agarose gel with MOPS buffer at 100 volts for 5 min. Lane M is 100 bp ladder. The fractions number 6, 7, 8 and 9 containing cDNA were collected and pooled.

An unamplified library was established by setting up three dilution of unamplified λ lysate and each dilution was tittered by calculation the number of plaque formation. The unamplified library information was shown in Table 7. The 1:5 and 1:10 dilution of unamplified λ lysate had 3 and 3.13 x 10^6 pfu/ml respectively which were acceptable for library construction. Percentage of recombinant clones from blue/white colony calculation was 99%. The 1:10 λ lysate dilution was selected to continue conversion from plaque to colony. The converted BM25.8 colonies were randomly picked up to determining the insert by colony PCR using SP6 and T7 primers. The products of colony PCR were checked on 2% agarose gel as shown in Fig. 35. DNA marker is 100 bp ladder plus 1.5 kb.

Table 7 The number of colony and the titer of 1:5, 1:10 and 1:20 unamplified λ diluted lysate

Dilution factor of Unamplified λ lysate	1:5	1:10	1:20
Number of colony	600	313	50
Titer (10^6 pfu/ml)	3	3.13	1

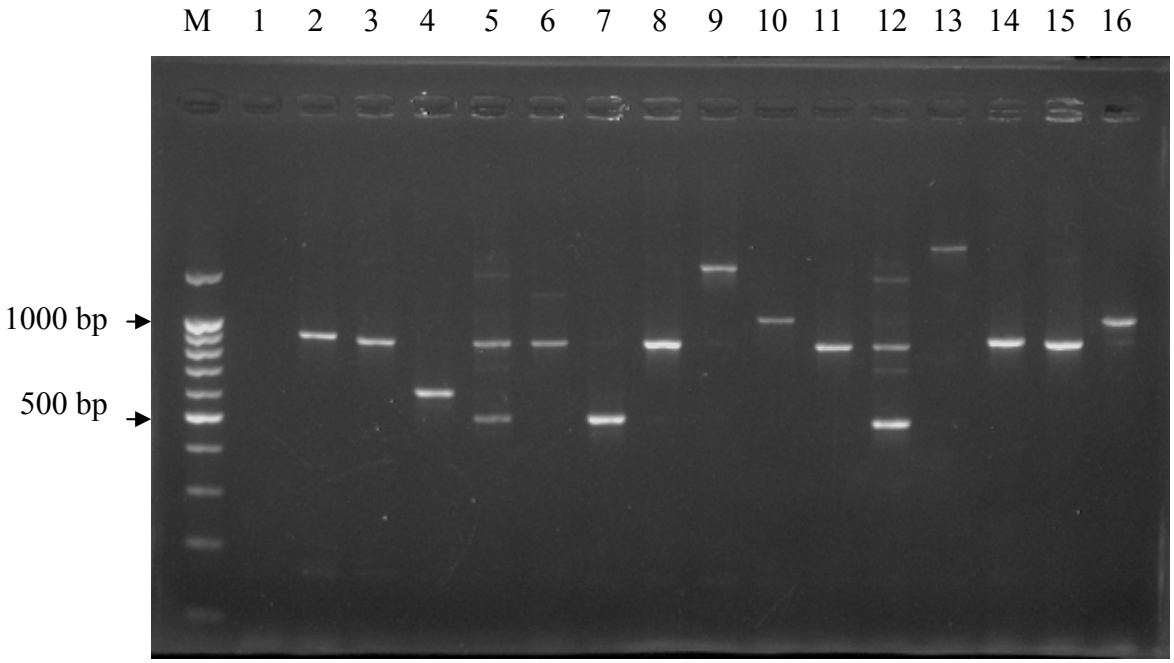

Figure 35 PCR products from colony PCR using SP6 and T7 primers separated by electrophoresed on 2% agarose gel with TAE buffer at 100 volts for 30 min. Lane M is 100 bp ladder.

The total number of colonies derived from an unamplified *M. rosenbergii* androgenic gland cDNA library was 1,100 colonies. A total of 1,000 clones were randomly picked up and purified the plasmids. Then, 960 clones were subjected to single-pass DNA sequencing, resulting in 776 (78%) EST sequences. The average read-length after vector clipping was 500 bp (in the range of 10-777 bp). The very short sequences (less than 50 bp) were not accepted. Then, 766 clones were further analysed. For identification of *M. rosenbergii* cDNA clones, the cutoff was set at an expected value ($E \leq 10^{-4}$). Identified clones generally shared a sequence identity >50% over a relatively long range >150 bp with the most similar sequence from BLASTX. Based on subjective criteria for gene identification at a minimum amino acid sequence similarity of expected value ($E \leq 10^{-4}$), 422 (55%) *M. rosenbergii* transcripts showed homology to previously described genes from *M. rosenbergii* and other species, including shrimp (Table 8). Five clones matching to the vector sequences were sorted out due to the non recombinant clone. ESTs matching known genes were categorized into seven categories on the basis of general functions

(storage protein 30.6%, gene/protein expression and modification 10.1%, hypothetical proteins 9.6%, cell/organism defense 8.3%, enzyme 2.1%, structural protein, cytoskeleton 0.5%, transport proteins 0.5% and other 3.3%) as shown in Fig. 36. In total, 42 known genes were represented and the percentage of transcripts in each category was calculated. Several transcripts matched previously-reported known proteins of *M. rosenbergii*, including alpha-2-macroglobulin, cortical rod-like protein and ferritin.

A total of 266 transcripts did not share significant sequence homology with any known sequence in the databases. Most of them are expected to be AGH or novel genes. Thus, the rests of no significant similarity sequences and some hypothetical proteins with unknown function were further analyzed by translating to protein sequences. After translation, some deduced amino acid sequences were too short to identify the protein and some could not present the start codon or frequently presented the stop codon. These sequences were not further considered. Therefore, the deduced amino acid sequences of 12 clones named B-H07, C-A010, E-A04, E-C04, E-H07, G-C011, I-C05, J-D03, A-D05 (CG31997-PA Tribolium), A-E05 (similar to CG17652-PA Tribolium castaneu) and F-D010 (carcinin-like protein) were further considered by SMART and multiple alignment with the pro-*Cq-IAG*, three pro-AGHs known in isopods (Manor *et al*., 2007; Ohira *et al*., 2003; Okuno *et al*., 1997) and internal peptide sequences from *de novo* sequencing were performed by ClustalW.

Table 8 The summary of *M. rosenbergii* androgenic gland cDNA library sequencing

Product	Species	Number of clones	E-value
No significant similarity found	-	266	-
26S protease regulatory subunit 6A	*Culex pipiens quinquefasciatus*	1	1.00E-89
26S proteasome regulatory complex subunit p50	*Drosophila melanogaster*	2	3.00E-95
40S ribosomal protein S23	*Dermacentor variabilis*	5	6.00E-76
40S ribosomal protein S27E	*Homarus americanus*	1	5.00E-43
60S ribosomal protein L30	*Lycosa singoriensis*	2	4.00E-45
60s ribosomal protein L39	*Ornithodoros parkeri*	1	4.00E-13
AGAP008475-PA	*Anopheles gambiae*	1	2.00E-46
alpha-2-macroglobulin	*Macrobrachium rosenbergii**	1	1.00E-44
anti-lipopolysaccharide AA-K isoform	*Litopenaeus vannamei**	1	1.00E-09
anti-lipopolysaccharide factor isoform 1	*Farfantepenaeus paulensis**	1	2.00E-25
anti-lipopolysaccharide factor like protein	*Marsupenaeus japonicus**	3	4.00E-25
cadmium metallothionein	*Palaemonetes pugio**	226	6.00E-04
calpain T	*Gecarcinus lateralis***	2	6.00E-107
carcinin-like protein	*Carcinus maenas***	4	2.00E-14
cathepsin D	*Penaeus monodon**	1	6.00E-107
Cofilin/actin-depolymerizing factor homolog (Protein D61) (Protein twinstar)	*Drosophila yakuba*	1	2.00E-14
conserved hypothetical protein	*Culex pipiens quinquefasciatus*	1	6.00E-107
copii-coated vesicle membrane protein P24	*Aedes aegypti*	1	2.00E-14
cortical rod-like protein	*Macrobrachium rosenbergii**	1	6.00E-107

Table 8 (Continued)

Product	Species	Number of clones	E-value
crustin	*Farfantepenaeus brasiliensis**	52	2.00E-14
cyclophilin-like protein	*Phlebotomus papatasi*	1	7.00E-65
cytochrome c oxidase polypeptide IV	*Bombyx mori*	2	2.00E-57
elongation factor-1 alpha	*Libinia emarginata***	3	6.00E-88
Eukaryotic initiation factor 1A	*Drosophila melanogaster*	3	4.00E-38
ferritin	*Macrobrachium rosenbergii**	7	1.00E-18
GA18544 gene product from transcript GA18544-RA	*Drosophila pseudoobscura*	1	8.00E-19
gamma subunit isoform 1	*Mus musculus*	1	4.00E-18
hypothetical protein	*Aedes aegypti*	2	1.00E-07
hypothetical protein	*Thermobia domestica*	23	9.00E-12
hypothetical protein CpipJ_CPIJ000746	*Culex pipiens quinquefasciatus*	16	4.00E-04
hypothetical protein LOC100119759	*Nasonia vitripennis*	13	4.00E-07
hypothetical protein LOC686222	*Rattus norvegicus*	2	1.00E-11
hypothetical protein LOC765150	*Strongylocentrotus purpuratus*	1	3.00E-13
intermediate filament protein C1	*Branchiostoma floridae*	1	7.00E-04
mitochondrial ATP synthase lipid binding protein	*Culex pipiens quinquefasciatus*	1	4.00E-07
mitochondrial cytochrome c oxidase subunit 6B	*Drosophila simulans*	3	3.00E-26
Mitochondrial Transcription Factor B1	*Drosophila melanogaster*	5	9.00E-12
peptidyl-prolyl cis-trans isomerase	*Aedes aegypti*	3	3.00E-64
PREDICTED: similar to CG17652-PA	*Tribolium castaneum*	3	6.00E-29
PREDICTED: similar to putative ribosomal protein L19e	*Strongylocentrotus purpuratus*	2	1.00E-26
putative ribosomal protein L27Ae	*Diaphorina citri*	3	1.00E-53

Table 8 (Continued)

Product	Species	Number of clones	E-value
Putative RNA polymerase II transcriptional coactivator	*Tetrahymena thermophila*	1	9.00E-18
putative serine proteinase inhibitor	*Pacifastacus leniusculus**	2	3.00E-24
ribosomal protein L10A	*Bombyx mori*	1	7.00E-86
ribosomal protein L14	*Gallus gallus*	1	4.00E-30
ribosomal protein L23	*Epinephelus coioides*	3	3.00E-62
ribosomal protein L44e	*Georissus* sp. APV-2005	2	1.00E-40
ribosomal protein LP1	*Argas monolakensis*	2	5.00E-21
ribosomal protein S15	*Danio rerio*	2	3.00E-36
ribosomal protein S15	*Poecilia reticulata*	1	3.00E-14
ribosomal protein S15	*Oncorhynchus masou formosanus*	1	7.00E-10
ribosomal protein S16	*Gallus gallus*	1	5.00E-66
ribosomal protein S27a	*Canis lupus familiaris*	1	1.00E-06
RNA polymerase small Zn-binding subunit,	*Aedes aegypti*	6	4.00E-18
SEC61, gamma subunit	*Mus musculus*	3	4.00E-18
similar to 60S ribosomal protein L36	*Ornithorhynchus anatinus*	1	2.00E-25
similar to apontic CG5393-PB, isoform B	*Apis mellifera*	1	8.00E-07
similar to ATPase inhibitor-like protein	*Nasonia vitripennis*	21	6.00E-15
similar to CG14235-PA, isoform A	*Tribolium castaneum*	7	2.00E-26
similar to Glycine cleavage system H protein,mitochondrial precursor (Pumpless protein)		1	4.00E-18

Table 8 (Continued)

Product	Species	Number of clones	E-value
similar to nudix (nucleoside diphosphate linked moiety X)-type motif 21	*Apis mellifera*	1	1.00E-98
similar to ornithine decarboxylase	*Nasonia vitripennis*	1	4.00E-21
similar to prefoldin 1	*Tribolium castaneum*	1	1.00E-05
similar to putative ribosomal protein L19e	*Strongylocentrotus purpuratus*	10	1.00E-26
similar to ribosomal protein S5	*Apis mellifera*	4	2.00E-65
similar to SCCA2/SCCA1 fusion protein	*Equus caballus*	1	1.00E-05
similar to ubiquinol-cytochrome c reductase complex7.2kDa protein isoform a	*Tribolium castaneum*	1	2.00E-12
SUMO-1-like protein	*Artemia franciscana*	5	3.00E-37
tail muscle elongation factor 1 gamma	*Procambarus clarkii*	2	2.00E-76
threonyl-tRNA synthetase	*Aedes aegypti*	1	3.00E-37
translation initiation factor 4C (1A)	*Anopheles gambiae*	1	2.00E-37
transmembrane protein 14C	*Bombyx mori*	1	3.00E-30
ubiquitin	*Portunus pelagicus***	1	2.00E-60
unnamed protein product	*Tetraodon nigroviridis*	1	1.00E-37
vitamin K epoxide reductase complex subunit 1 precursor	*Ixodes scapularis*	2	2.00E-36
Total		761	

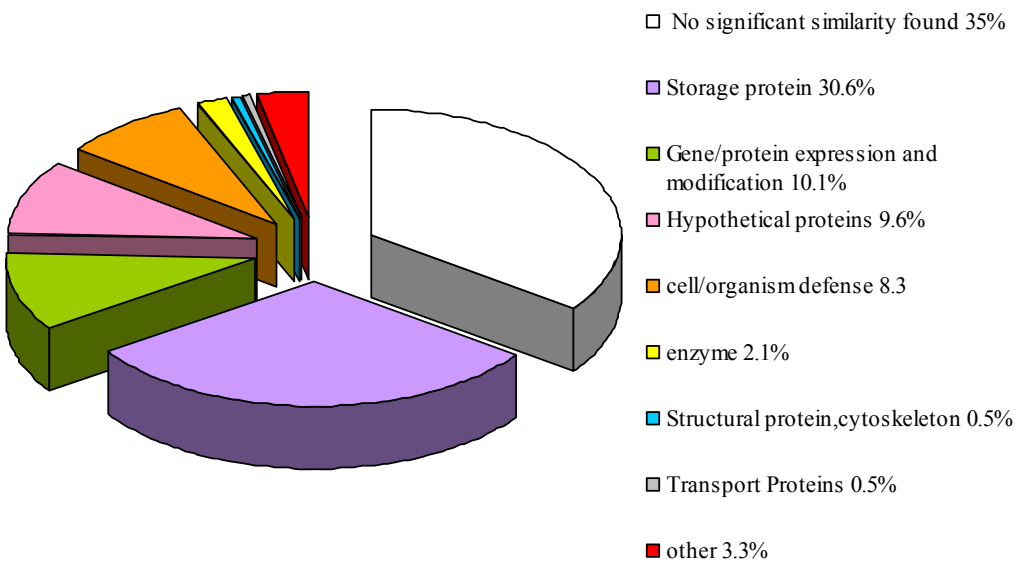

Figure 36 Sequence similarity analysis of 761 ESTs (61 putative genes) from *M. rosenbergii* androgenic gland cDNA library by BLASTX. More than 35% of the sequences had no significant similarity (E-value > 0.0001) to any Uniprot protein. 9.6% were similar to hypothetical proteins, and the rest could be related to proteins associated with protein in the NCBI database.

The SMART domain search tool predicted the existence of a domain between residues 22-84 and 27-71 of C-A010 similar to insulin growth factor-binding protein (E value= 2.46e+03) and to Laminin-type epidermal growth factor-like domain (E-value = 9.44e+02), respectively. The residues 24-64 and 66-105 of F-D010 were similar to insulin growth factor-binding protein (E value= 3.39e+03) and to insulin/insulin-like growth factor/relaxin family (E-value = 2.17e+03), respectively (Fig. 37). The deduced amino acids and theoretical molecular weight of C-A010 and F-D010 calculated by ProtParam program (ExPASy Proteomics Server) are 134 amino acids/15 kDa and 111 amino acids /12.2 kDa, respectively. However, there was no deduced amino acid sequence from any clone which ClustalW matching score with the pro-*Cq-IAG*, three pro-AGHs known in isopods and internal peptide sequences from *de novo* sequencing was higher than 20%. Besides that, the deduced amino acid sequence derived from products of primers designed from *A. vulgare*, *P. scaber*, *P.*

dillatatus AGH and of *C. quadricarinatus* insulin-like androgenic gland factor were no similarity to any deduced amino acid sequence from 12 library clones and internal amino acid sequences from de novo sequencing significantly.

Since F-D010 was similar to insulin/insulin-like growth factor/relaxin family which included AGH, the structure of F-D010 deduced amino acid sequence was determined by prediction from bioinformatic tools. The SignalP 3.0 result showed the existing of 16-amino acids signal peptide with max cleavage site probability 0.507 while TargetP 1.1 result revealed that this signal peptide involved the secretory pathway with reliability class 2 (almost strongest prediction). The predicted signal peptide sequence of F-D010 has some sequence similarity to the *A. vulgary*, *P. scaber* and *P. dillatatus* AGH and *Cq-IAG* signal peptides (25, 12, 12 and 6%, respectively) No site of N-glycosylation was predicted in this sequence. There were 12 Cys residues in their sequences which were no prediction of disulfide bond forming.

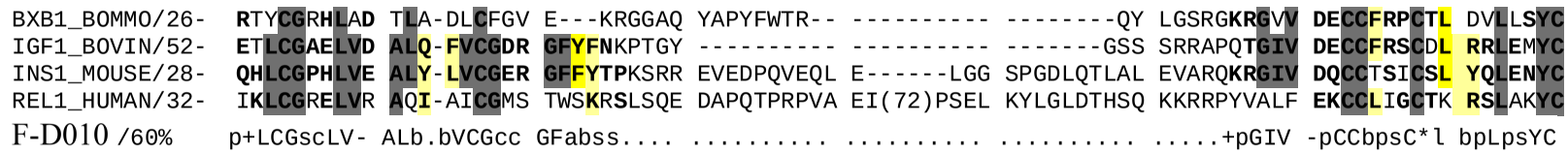

Figure 37 Multiple sequence alignment of the residues 66-105 of F-D010 with representative members of the insulin/insulin-like growth factor/relaxin family, by SMART domain search. The sequences shown include: mouse insulin [INS1_MOUSE, (Wentworth *et al*., 1986)], insulin-like growth factor I of bovine [IGF1_BOVIN, (Fotsis *et al*., 1990)], human relaxin H1 [REL1_HUMAN, (Hudson *et al*., 1984)], bombyxin C-1 of *B. mori* [Silk moth, BXA6_BOMMO, (Nagasawa *et al*., 1986)], Conserved Cys residues are shown in boxes while the (-) indicates gaps introduced into the amino acid sequence to allow for the maximal degree of identity in the alignment.

3. Androgenic gland hormone bioactivity determination

Two microliters of the acid-alcohol fraction, acetone extract of the androgenic tissues and each pooled fraction from Sep-Pak C18 column were injected into 20-30 days old *M. rosenbergii* females at the position of the first ventrally abdominal segments by microsyringe. Eight injections were given, each at 10 day intervals, with the prawns being sacrificed ten days after the final injection. After 80 days of injections, the animals were observed for indications of masculinization or inhibition of feminine trait. The result of bioactivity determination was presented in Table 9

Table 9 Survival of the musculinized *Macrobrachium rosenbergii* at 80 days after the injection

group	Sample of injection	Initial number	Survivors (at 80 days)	Musculinized female
control	Distilled water	7	2	0
1	Acid-alcohol fraction	7	0	0
2	Acetone precipitated fraction	7	0	0
3	unbound	7	1	0
4	20% acetonitrile	7	0	0
5	40% acetonitrile	7	1	0
6	60% acetonitrile	7	0	0

At 80 days after injection, 2 of 7 postlarvae in control group survived while there was only 1 of 7 survived in the injection of 20% acetonitrile and 60% acetonitrile fraction. However, none of the survivors were successfully reversed to male. The external male characteristics such as appendix muculina, male gonopore, body size and appendages length could not be observed.

Discussion

In this study, a new androgenic gland hormone, AGH has been discovered in the androgenic tissues of male *M. rosenbergii*. Some preliminary properties investigated in this study have been published (Narksen *et al.,* 2006; 2007). In this thesis, two major parts of the result will be discussed. Firstly, the approach to purify and structural characterize of AGH will be discussed. This will be followed by a discussion of the molecular cloning of the AGH from cDNA.

1. Purification of AGH

Due to the expectation that AGH of the freshwater prawn may present in a specific tissue, the androgenic tissues and vas deferens were isolated from the prawn to be sources for protein extraction. Since AGH of an isopod crustacean, *A. vulgare* has been reported to be a peptide of molecular mass 11,000 by MALDI-TOF mass spectrometry (Martin *et al*., 1999), so the acid-alcohol was chosen to extract the prawn tissues to remove high molecular size proteins out of the samples. Then the low molecular weight proteins including the AGH were obtained by acetone precipitation. RP-HPLC has been widely employed to separate many peptides, based on its principle of the hydrophobic interactions between a polar solvent, non-polar side-chain of the peptides, and the non-polar stationary phase. By using Sep-Pak C18 cartridge, peak resolution of the chromatogram was not satisfied (Fig. 17). However, it can remove most proteins from the extract of androgenic tissues as unbound fractions. The MALDI-TOF MS spectrum of the bound fractions which were eluted by various concentration of acetronitrile gave information of a different intense peak of 10.2 kDa. In order to improve the peak resolution of the RP chromatography, the separation was performed by using Lichrosorb HPLC column. Unfortunately, no well separated peaks were observed (Fig.23). However, the 42.5% acetronitrile eluted fraction from androgenic tissues extract showed a unique peak of 10.2 kDa in MALDI-TOF MS spectrum, which was not observed in the vas deferens extract (Fig. 24). The result indicates that this 10.2 kDa protein is specifically localized in the androgenic gland and is not present in the vas deferens of the prawn.

The purified recombinant AGH from *A. vulgare* showed a complex structure, consisting of two peptide chains (A chain and B chain) which connected by disulfide bonds (Okuno *et al*., 2002) and a chain carried a N-glycosylated moiety (Greve *et al,* 2004). So in this experiment, the purified 10.2 kDa protein from RP-HPLC was investigated for the presence of disulfide bond and glycan moiety by following the reduction of the AGH molecular mass after treated with a reducing agent (DTT) and a deglycosylated enzyme (PNGase F), repectively. The result of unchanges in the molecular mass (Fig. 25) indicated that the purified AGH of freshwater prawn is a single polypeptide that does not have any disulfide bond and glycan in its molecule.

The peptide mass fingerprint of the purified 10.2 kDa AGH was determined in comparison to those AGH of various crustacean species in the NCBI database. The result of no matching of the purified 10.2 kDa AGH with any crustacean AGHs reveal that AGH of the freshwater prawn is quite different from the former reported AGH.

2. Molecular cloning of AGH from cDNA.

2.1 Partial cloning of the AGH cDNA

Cloning of the AGH cDNA was established using primers, designed based on the nucleotide sequences of AGHs from *A. vulgare*, *P. scaber*, *P. dillatatus* and insulin-like androgenic gland factor of *C. quadricarinatus*. Although the amplification gave various PCR products, the nucleotide sequences of these PCR products showed no similarity to AGH from *A. vulgare*, *P. scaber*, *P. dillatatus* and *C. quadricarinatus* insulin-like androgenic gland factor. The comparison of amino acid sequences of the AGH from *P. scaber* and *P. dillatatus* to AGH from *A. vulgare* revealed very high identity of the precursors (65 and 63 %, respectively) and the mature hormone (72-82%) (Ohira *et al.*, 2003). The similar results have been reported among the same order group of isopods species, whereas the comparison with other species such as decapods showed a decrease in similarity. Multiple sequence alignment of *C. quadricarinatus* insulin-like androgenic gland factor predicted precursor, with the

three AGH precursors of isopods (Fig.7) showed that *C. quadricarinatus* insulin-like androgenic gland factor has some sequence similarity (between 16 and 19% identity) to the three already known isopod AGHs (Manor *et al*., 2006). However, the regions in the B and A chains of the 3-D model of *C. quadricarinatus* insulin-like androgenic gland factor were found to have high similarity with insulin family members and the AGH in isopods especially with bombyxin (53.8% identity for the region between the two Cys residues in the B chain, and 37.5% identity for the entire region of the A chain). Nevertheless, *C. quadricarinatus* insulin-like androgenic gland factor was not confirmed to be the same protein as AGH, the exclusive localization of its expression to the androgenic gland was confirmed. Based on our literature search, there was no report of amino acid sequence identity between the lower and higher crustacean species. Therefore, the deduced amino acid derived from the nucleotide sequence obtained from PCR products amplified from *M. rosenbergii* androgenic gland cDNA showed no similarity to the amino acid sequences of the AGHs from *A. vulgare*, *P. scaber*, *P. dillatatus* and insulin-like androgenic gland factor of *C. quadricarinatus*. The primers used may be not specific.

To overcome this problem, primers designed from amino acid sequences of *M. rosenbergii* AGH must be used. In this experiment such primers were derived from 9.7, 10.7, 12.5, 13.6, 10, and 15 kDa protein band on Tricine-SDS gel of the acetone extract of the prawn androgenic gland, whose molecular sizes were nearly 10.2 kDa (Fig. 29). Tryptic peptide fragments from each protein band were de novo sequenced by Q-TOF-MS. Both forward and reverse primers were designed from each amino acid sequence. The resulted PCR products will be sequenced for further establishing the full length of AGH cDNA by RACE technique.

2.2. *M. rosenbergii* androgenic gland cDNA library

The generation of ESTs has proven to be a useful and rapid method to identify and isolate large number of expressed sequences. 776 ESTs of *M. rosenbergii* androgenic gland were analysed. One-pass sequencing from the 5′ end was applied to 776 independent colonies. The length of nucleotide sequences of the 776 readings was

in the range of 10-777 bp with poly A tail. Since the technical problem occurred with 3′ end sequencing (data not shown), the sequences did not present the poly A tail at 3′ end. After removal of vector-based sequences, 766 readings (98.7%) were analyzed further. After eliminate the plasmid vector sequence, the EST sequences shorter than 150 bp were discarded before homology searches because algorithm did not worked well with the short query sequence. The cDNA library had average insert sizes of 500-700 bp. For identification of *M. rosenbergii* cDNA clones with BLASTX, the cutoff was set at an expected value ($E \leq 10^{-4}$). Identified clones generally shared a sequence identity more than 50% over a relatively long range more than 150 bp with the most similar sequence from BLASTX. Based on subjective criteria for gene identification at a minimum amino acid sequence similarity of expected value, 422 (55%) *M. rosenbergii* transcripts showed homology to previously described genes from *M. rosenbergii* and other species, including shrimp. ESTs matching with known genes were categorized into seven categories on the basis of general functions. While a total of 266 transcripts did not share significant sequence homology with any known sequence in the databases. Since AGH in *M. rosenbergii* had not been identified, most of transcripts are expected to be AGH or novel genes. Manor *et al*. (2007) constructed the subtractive cDNA library from an androgenic gland of the crayfish *Cherex quadricarinatus*. *Cq-IAG* was found from 300 DNA sequence fragments and more than 50% of the assembled sequences share no significant similarity (E value > 0.01) to any known genes or proteins in the database, while 7% of the assembled sequences share significant similarity with genes encoding hypothetical proteins with no annotated function. The number of library clones in this study was more than that in *Cherex quadricarinatus* about three times The reason that all clones in this study were from unamplified library made the difficulty of novel low-copy gene discovery. Moreover, subtractive cDNA library make ease for identification of the tissue-specific gene such as *Cq-IAG*. Interestingly, 226 ESTs (30% of total ESTs) were similar to *Palaemonetes pugio* cadmium metallothionein which indicates the cadmium-contaminated environment of giant freshwater prawn farming. In this present study, this is the first report of *M.rosenbergii* androgenic gland cDNA library information.

The alignment among the deduced amino acid of 12 library clones of cDNA amplified using primers designed from *A. vulgare*, *P. scaber*, *P. dillatatus* AGH and *C. quadricarinatus* insulin-like androgenic gland factor and internal peptide sequences from *de novo* sequencing showed no similarity. However, the deduced amino acid sequences from 12 library clones generatedthe putative genes which need to be further identified and studied their function, interestingly.

The SMART domain search tool prediction showed some interesting information. Though, the deduced amino acid sequences of C-A010 and F-D010 clones matched to the protein domain database with low E- value scores. Interestingly, the similarity F-D010 to insulin/insulin-like growth factor/relaxin family was as same as the pro-*Cq-IAG* and three pro-AGHs in isopods (Manor *et al*., 2007; Ohira *et al*., 2003; Okuno *et al*., 1997). The comparison of the amino acid sequences of the AGH precursor from *P. scaber* and *P. dillatatus* with that from *A. vulgare* revealed that the sequence identities were very high (65 and 63 %, respectively) as same as the identities of the mature hormone (72-82%) (Ohira *et al.*, 2003). This comparison was established among the same order group, isopod species, whereas the comparison with other order species such as decapods may decrease the similarity (Table 10). Multiple sequence alignment of *C. quadricarinatus* insulin-like androgenic gland factor predicted precursor, with the three AGH precursors of isopods showed that *C. quadricarinatus* insulin-like androgenic gland factor has some sequence similarity (between 17 and 18% identity) to the three already known isopod AGHs (Manor *et al*., 2006) as shown in Table 10. However, the regions in the B and A chains on which the 3D model of *C. quadricarinatus* insulin-like androgenic gland factor is based, were found to have high similarity with insulin family members as same as the AGH in isopods and especially with bombyxin (53.8% identity for the region between the two Cys residues in the B chain, and 37.5% identity for the entire region of the A chain). F-D010 deduced amino acid sequence has some sequence similarity to the *P. scaber*, *P. dillatatus* and *A. vulgary* AGH and *Cq-IAG* (8, 10, 14 and 17%, respectively) as shown in Table 10. The difference in structure occurred by species evolution might be feasible.

Table 10 Similarity of amino acid sequences of *P. scaber*, *P. dillatatus* and *A. vulgare* AGH precursors, pro-*Cq*-IAG and F-D010

	A. vulgare	*P. dillatatus*	*P. scaber*	*Cq*-IAG
P. dillatatus	63%	-	-	-
P. scaber	65%	87%	-	-
Cq-IAG	18%	17%	17%	-
F-D101	14%	10%	8%	17%

Characteristics of *P. scaber*, *P. dillatatus* and *A. vulgare* AGH precursors, pro-*Cq*-IAG and F-D010 were shown in Table 11. Although F-D010 is similar to insulin/insulin-like growth factor/relaxin family, its amino acids and molecular weight are quiet different from *P. scaber*, *P. dillatatus* and *A. vulgare* AGH precursors and pro-*Cq*-IAG. This may be a reason for the low E-value of SMART result.

Table 11 Summary of the *P. scaber*, *P. dillatatus* and *A. vulgare* AGH precursors, pro-*Cq*-IAG and F-D010

	AGH of *A. vulgare*	AGH of *P. scaber*	AGH of *P. dillatatus*	*Cq*-IAG	F-D101
Nucleotide (bp)	649 (432)	672 (435)	672 (438)	1445 (528)	-
Precursor (aa / kDa)	144 / 16.5	145 / 16.2	146 / 16.2	176 / 19.9	111 / 12.2
Signal peptide (aa)	21	21	21	23	17
Mature protein (aa / kDa)	73 / 10.7	75 / 10.4	75 /10.4	82 / 9.88	-
B chain (aa)	44	44	44	36	-
C peptide (aa)	46	45	46	71	-
A chain (aa)	29	31	31	46	-
N-glycosylation	Asn18 (A chain)	Asn18 (A chain)	Asn18 (A chain)	Asn53, Asn 137	None
Intra-chain disulfide bond	2 (A,B chain)	2	2	1 (A chain)	-
Inter-chain disulfide bond	2	2	2	2	-

However, further experiments such as tissue-specific determination by RT-PCR or southern blot analysis are required in order to examine tissue-specificity of these sequences. Finally, the biological activity assay of this protein should be further investigated because it is the only way to confirm the being of AGH.

3. Androgenic gland hormone bioactivity determination

To confirm the sex reversal activity of the purified AGH, each acetronitrile eluted fraction from Sep-Pak C 18 catridge was neutralized and injected into the young female prawns, and indications of masculinization were followed for 80 days (Table 8). This method is not successful because the number of survival prawns was very low, even in the control group. The examples of successful in vivo biological

assay of the isopod AGH have been reported in *A. vulgare* (Okuno *et al*., 2002) and *P. scaber* (Greve *et al*, 2004). The reason of those successful may be because that the response of musculinization can be recognized within a short period, 8-10 days after the hormonal injection. In this experiment, much longer period of the musculinized responses was required. Thus microbial infection may occur to the prawns via the wound or the injury by the injection. The increase in number of the experimental young prawns and more careful in the method of injection as well as well culturing environment could be done to solve the problem.

CONCLUSION

1. *Macrobrachium rosenbergii* AGH is purified by acid alcohol precipitation. The protein contents in the androgenic gland and vas deferens were 31 and 38 µg per mg tissue, respectively.

2. The RP-HPLC-MALDI was used to analyze the protein profile of androgenic tissues and vas deferens. The protein eluted by 42.5% acetronitrile with a molecular mass of 10.2 kDa was suggested to be the AGH. This protein did not contain glycan moiety and disulfide bridges between 2 peptide chains.

3. The peptide mass fingerprint of 10.2 kDa protein did not show any matching to AGHs available in the NCBI database. Our AGH may differ from the numerous crustacean AGHs identified and characterized to date.

4. Partial cDNA fragment of AGH was amplified using primers based on the nucleotide sequence of the AGH from *Armadillidium vulgare*, *Porcellio scaber*, *P. dillatatus* and *Cherex quadricarinatus*. No nucleotide sequences of these PCR products were similar to AGHs from *A. vulgare*, *P. scaber*, *P. dillatatus* and *C. quadricarinatus* insulin-like androgenic gland factor.

5. SDS-PAGE analysis of protein isolated from androgenic gland showed the proteins with molecular mass of 9.7, 10.7, 12.5, 13.6 and 15 kDa. The amino acid sequences of each protein were determined by LC-MS/MS. Partial cDNA fragments of AGH cDNA amplified using primer designed from amino acid sequences were then cloned and the DNA sequences of the DNA fragments were determined.

6. 776 clones from an unamplified *M. rosenbergii* androgenic gland cDNA library were sequenced from 5′ end. 226 ESTs were unknown (35%) and the other matcing with known gene were categorized into seven categories on the basis of general functions which were storage protein (30.6%), gene/protein expression and

modification (10.1%), hypothetical proteins (9.6%), cell/organism defense (8.3%), enzyme (2.1%), structural protein, cytoskeleton (0.5%), transport proteins (0.5%) and other (3.3%).

7. The residues 66-105 of F-D010 from androgenic gland cDNA library found from 761 unamplified clones was similar to insulin / insulin-like growth factor / relaxin family from SMART domain search. The similarity of deduced amino acid sequence to the *A. vulgary* AGH and *Cq-IAG* were 14 and 17, respectively.

8. The biological activity of the purified AGH from *M. rosenbergii* was determined by directly injection into postlarvae females. This bioassay was not successful. However, the biological activity assay of this protein should be further investigated because it is the only way to confirm the being of AGH.

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APPENDIX

1. Reagent for molecular cloning

50X TAE buffer (Tris-Acetate-EDTA)

Tris base 242 g
Acetic acid 57.1 ml
0.5 M EDTA 100ml
Add distilled water 1 litre and adjust pH to 8.5.

Chloroform : Isoamyl alcohol (24 : 1, v/v)

Chloroform (Merck) 24.0 ml
Isoamyl alcohol 1.0 ml
Both reagents were mixed together and stored in a dark bottle at room temperature.

Ethidium bromide (10 mg/ml)

Ethidium bromide 1.0 g
Distilled water 10.0 ml
The solution was stored in a dark bottle at room temperature.

6X gel-loading dye buffer

0.25 % bromophenol blue,
0.25 % xylene cyanol FF
30 % glycerol in water
The chemicals were dissolved and adjusted the final volume with distilled water.

DNA Tracking Dye

Glycerol	500 µl
0.1 M EDTA	200 µl
1 % xylene Cyanol FF	60 µl
1 % Bromophenol blue	60 µl
1 M Tris HCl, pH 7.5	180 µl

1 M IPTG

Isopropylthio-β-D-galactoside 2.38 g

Distilled water 100 ml

The solution was sterilized by filtration through a 0.2 ๓m filter and dispensed the solution into 1 ml aliquot tube and stored at –20 °C.

20 mg/ml X-gal

5-bormo-4-chloro-3-indolyl-β-D-galactoside 100 mg

The chemical was dissolved in 2 ml of dimethyl-formamide. The solution was stored in a tube covered with aluminum foil and stored at -20 °C.

2. Media for bacterial culture

Luria-Bertani medium (LB medium per liter)

Tryptone (Difco)	10 g
Yeast extract (Difco)	5.0 g
NaCl	10 g

Adjust pH to 7.0 with NaOH. Then the solution was adjusted to the final volume of 1000 ml with distilled water and sterilized by autoclaving.

LB plates with ampicillin

Fifteen grams of agar was added to 1 lite of LB medium then the media was sterilized by autoclaving. The medium was allowed to cool to 50 °C before adding ampicillin to a final concentration of 100 μg/ml. The medium (20-25 ml) was pouredinto 85 mm petri dishes. The agar was allowed to harden. Agar plates were stored at4 °C for up to 1 month or room temperature for up to 1 week.

LB plates with ampicillin/IPTG/X-gal

The LB with ampicillin was prepared then supplement with 0.5 mM IPTG and 80 μg/ml X-Gal and pour the plates. Alternatively, 100 μl of 100 mM IPTG and 20 μl of 50 mg/ml X-gal may be spread over the surface of an LB ampicillin plate and allowed to absorb for 30 minutes at 37 °C prior to use.

3. Reagents for Tricine-SDS PAGE

Gel monomer (30% acrylamide, 29:1).

acrylamide 29.0 g
bisacrylamide 1.0 g
dissolved in a total volume of 100 ml deionized water.

Gel Buffer

Tris base 181.7 g
SDS 1.5 g

Dissolve in 400 ml deionized water. Adjust the pH to 8.45 using concentrated HCl. Bring to a final volume of 500 mL with deionized water. Store at 4 °C.

Anode Buffer (Lower Buffer)

Tris base 12.11 g

Dissolve in 400 ml deionized water. Adjust the pH to 8.9 using concentrated HCl. Bring to a final volume of 500 mL with deionized water. Store at 4 °C.

Cathode Buffer (Upper Buffer)

Tris base	6.055 g
Tricine	8.96 g
SDS	0.5 g

Dissolve in 500 ml deionized water

NOVEX 2X Tricine-SDS sample buffer

3.0 M Tris-HCl, pH 8.45	3.0 ml
Glycerol	2.4 ml
SDS	0.8 g
0.1% Coomassie Blue G	1.5 ml
0.1% Phenol Red	0.5 ml
Distilled water	to 10 mL

Separating Gel: 15 % running gel for four Novex 1.0 mm mini-gel cassettes

gel monomer	15.0 ml.
gel buffer	10 ml
glycerol	3.1 ml
deionized water	4.8 ml
TEMED	40 uL
10% ammonium persulfate	0.1 ml

Stacking Gel: 4.0 % stacking gel for four Novex 1.0 mm mini-gel cassettesdH

gel monomer	1.25 ml
gel buffer	2.5 ml
water	6.25 ml
10% ammonium persulfate	0.05 ml
TEMED	20 uL

4. Reagents for in gel digestion

Wash solution

Methanol	10 ml
Water	5 ml
acetic acid	1 ml

adjust the total volume to 20 ml with water

100 mM ammonium bicarbonate

ammonium bicarbonate	0.2 g
water	20 ml

50 mM ammonium bicarbonate

100 mM ammonium bicarbonate	2ml
Water	2ml

10 mM DTT

Dithiothreitol	l0.0015 g
100 mM ammonium bicarbonate	1.0 ml

100 mM iodoacetamide

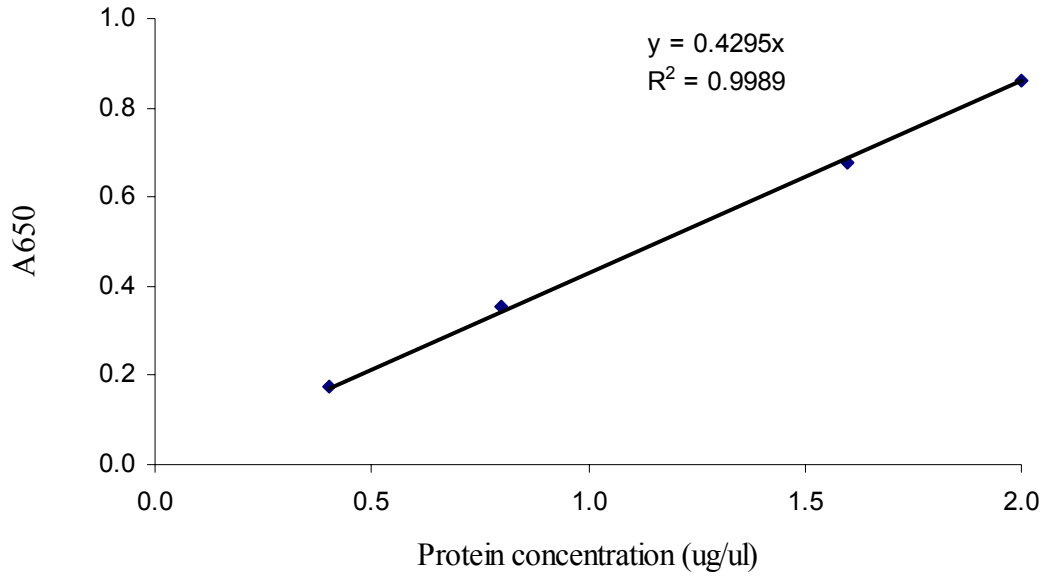
Iodoacetamide	0.018 g
100 mM ammonium bicarbonate	1 ml

Extraction buffer: (50% acetronitrile and 0.1% TFA)

Acetonitrile	5 ml
Water	5 ml
TFA	10 µl

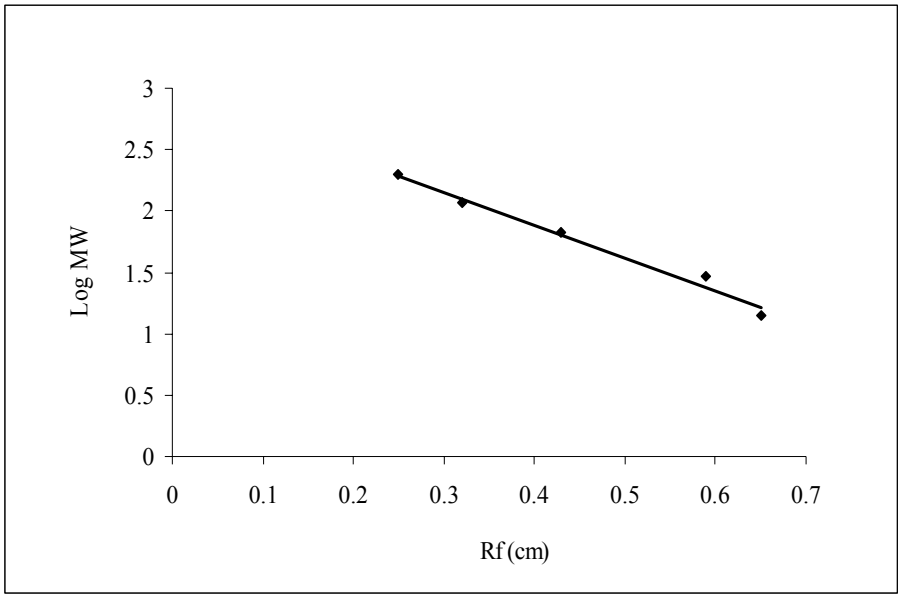
Adjust he total volume to 20 ml with water

5. Determination of Protein concentration



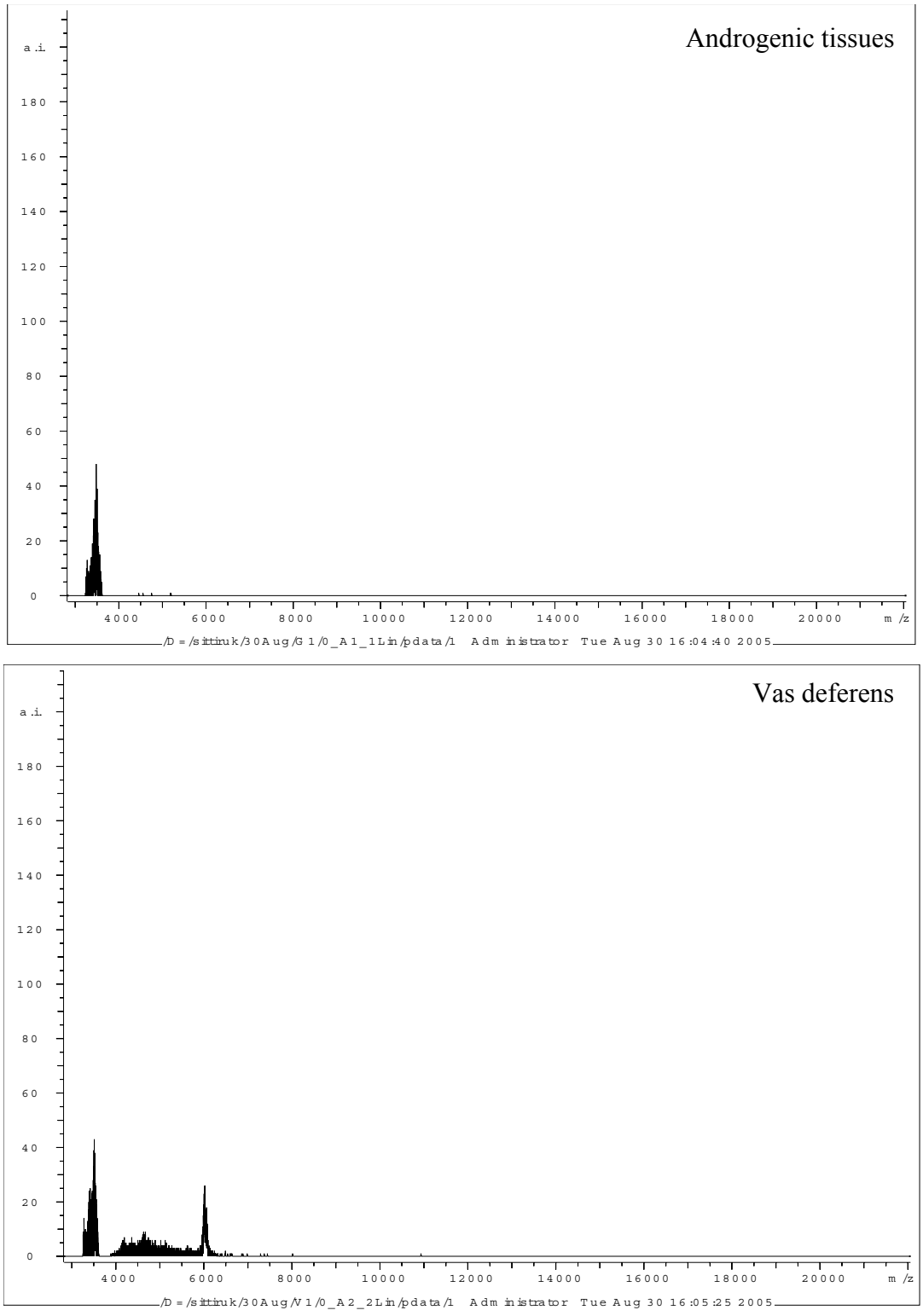
Appendix Figure 1 Standard curve of bovine serum albumin (BSA) by Lowry's method

6. Standard of protein molecular weight markers

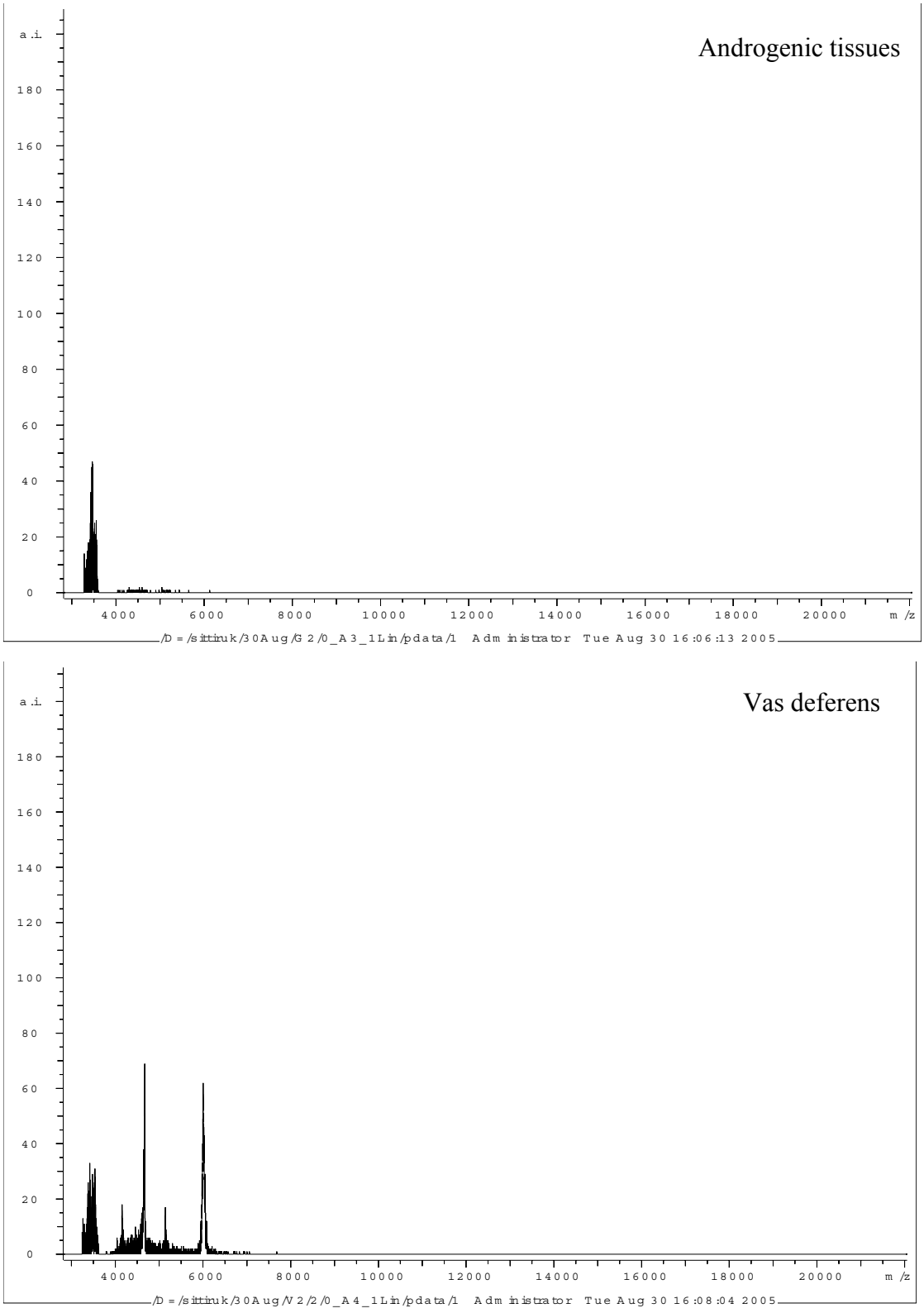


Appendix Figure 2 Standard curve of protein molecular weigh markers

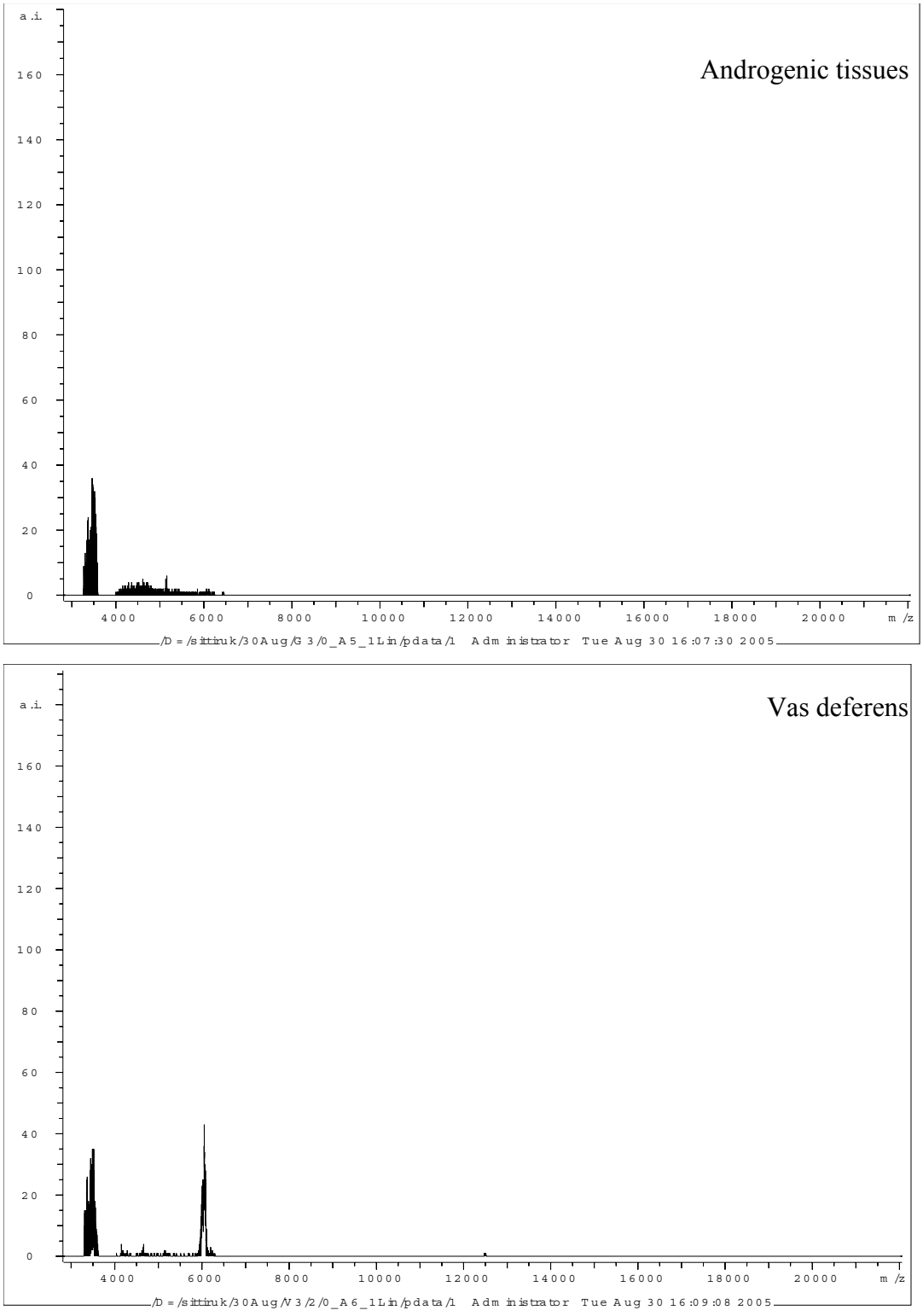
7. RP HPLC-MALDI analysis of each fraction from Licrosorb column



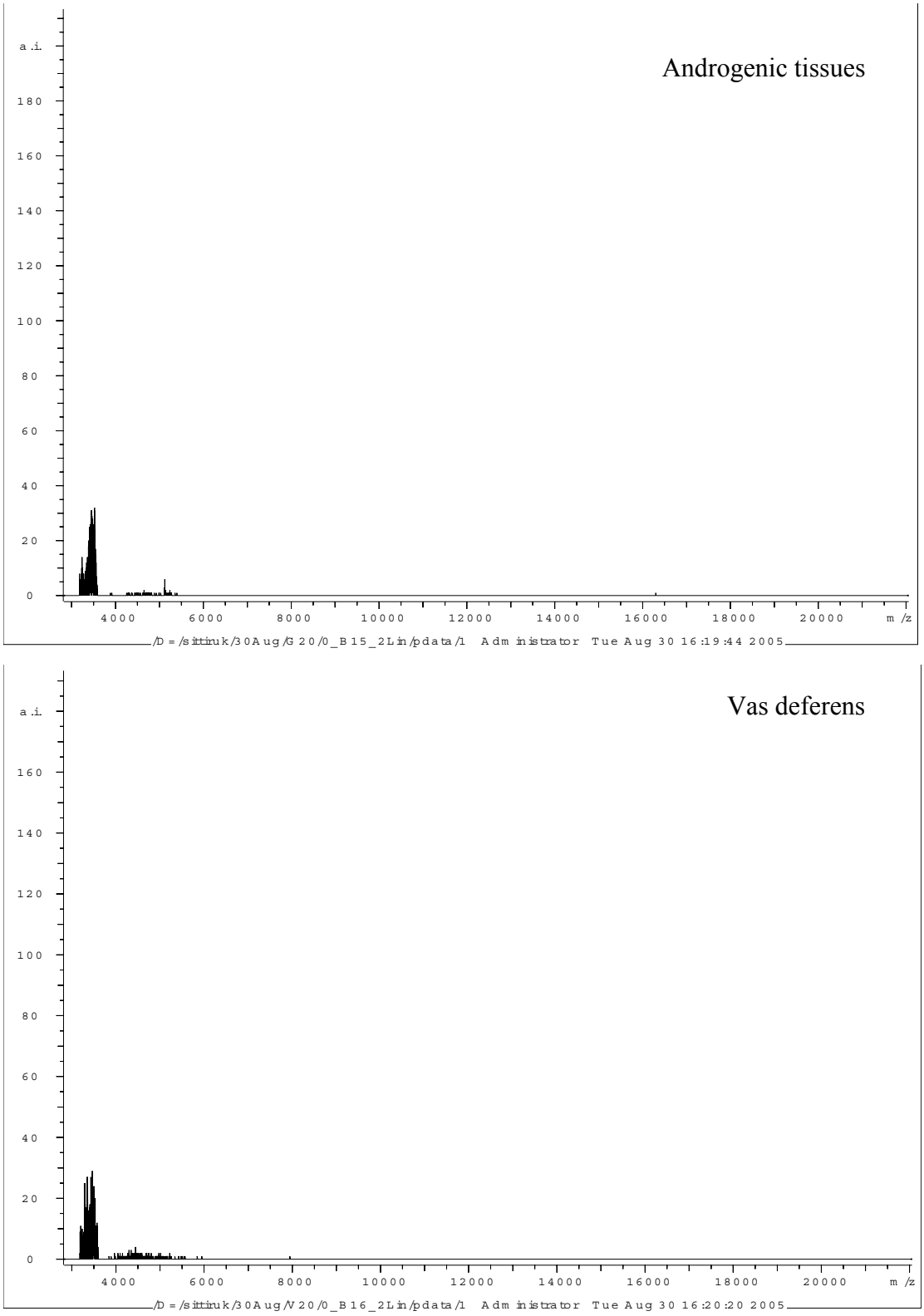
Appendix Figure 3 Fraction number 1 at 1 min (eluted by 0.1% TFA)



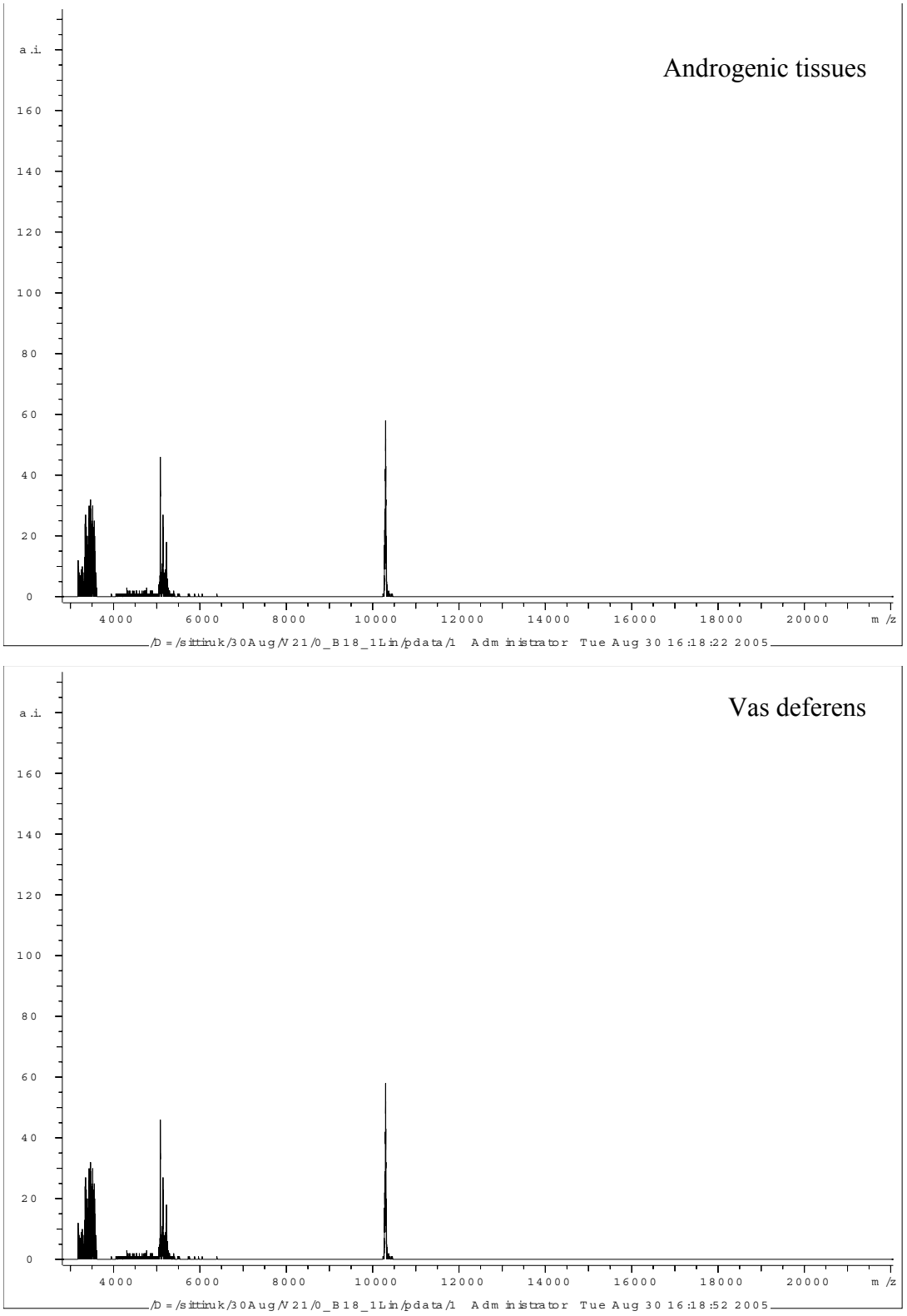
Appendix Figure 4 Fraction number 2 (eluted by 0.1% TFA)



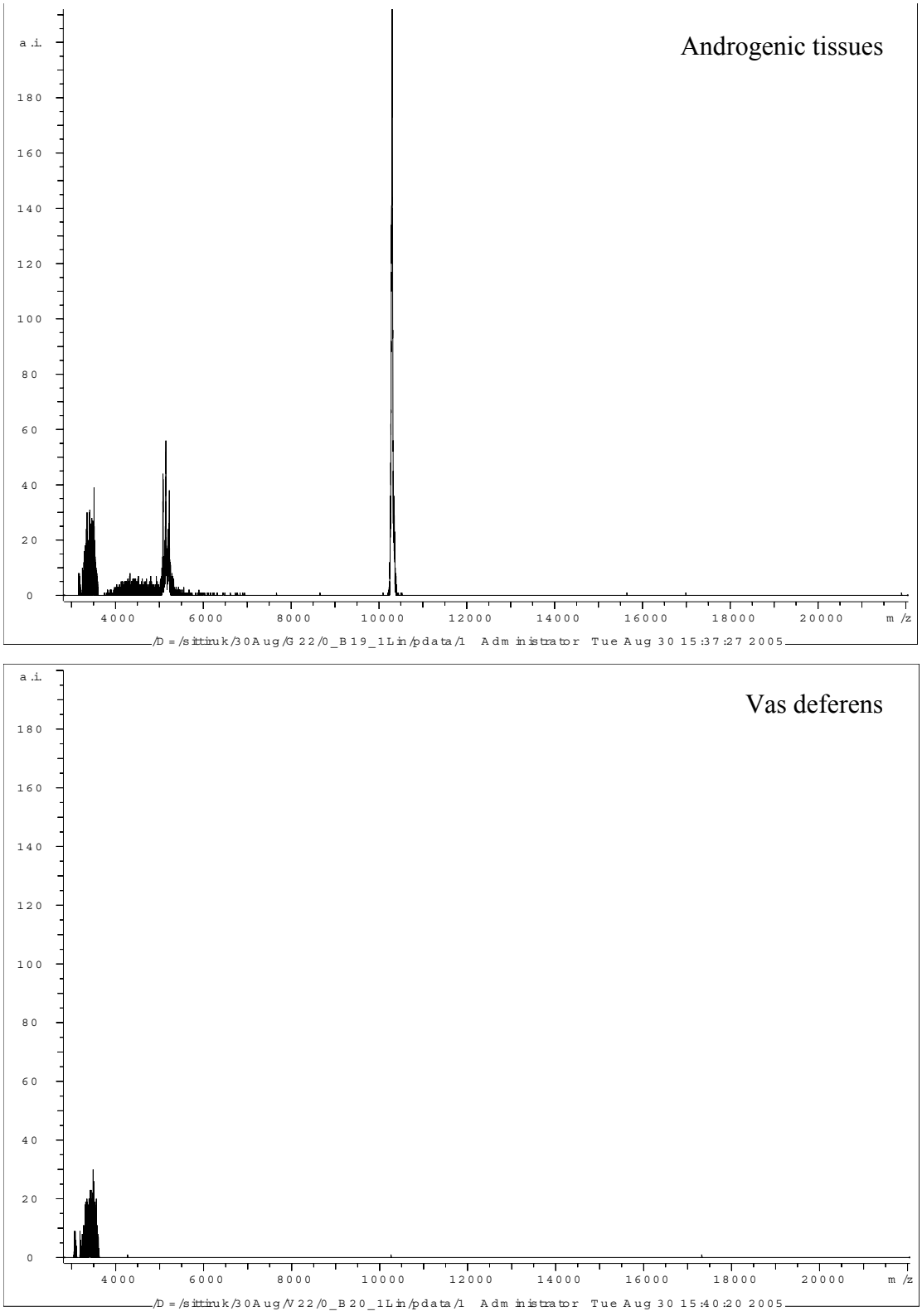
Appendix Figure 5 Fraction number 3 (eluted by 0.1% TFA)



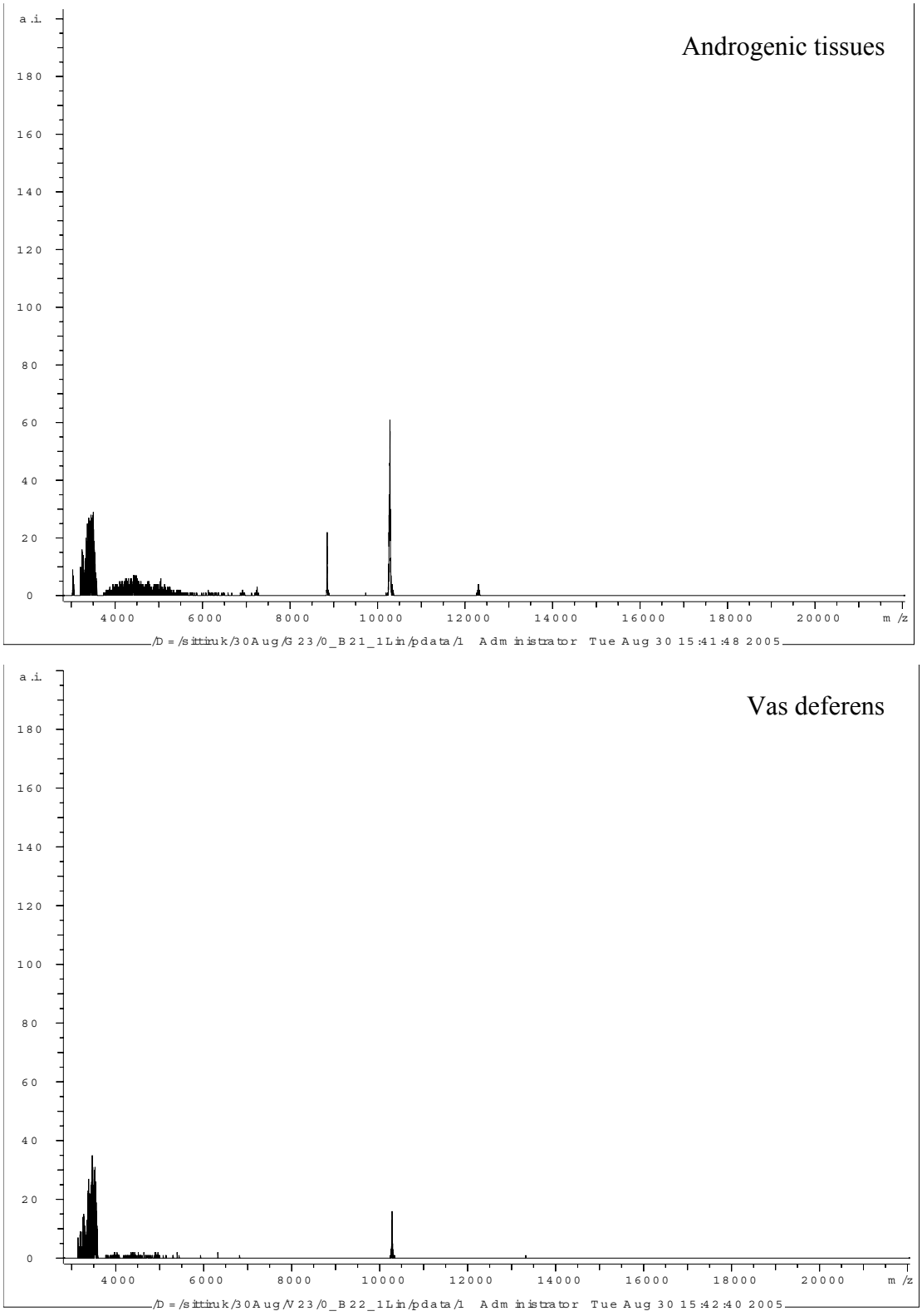
Appendix Figure 6 Fraction number 20 (eluted by 37.5% acetronitrile in 0.1%TFA)



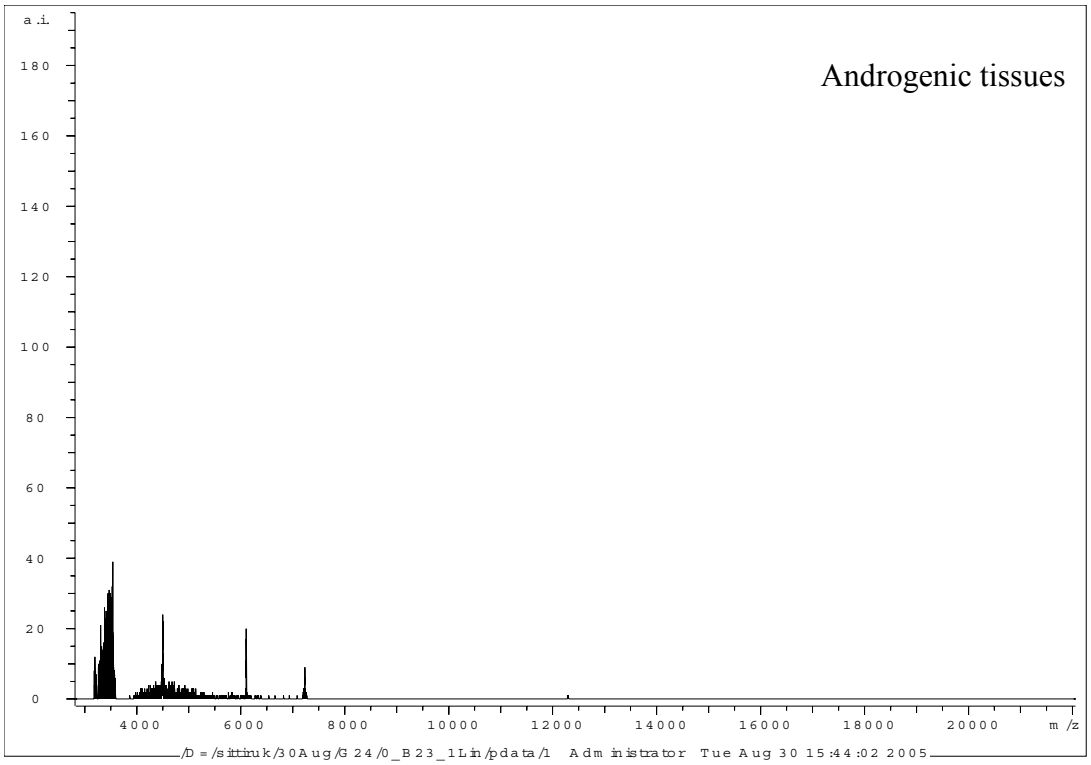
Appendix Figure 7 Fraction number 21 (eluted by 40% acetronitrile in 0.1%TFA)

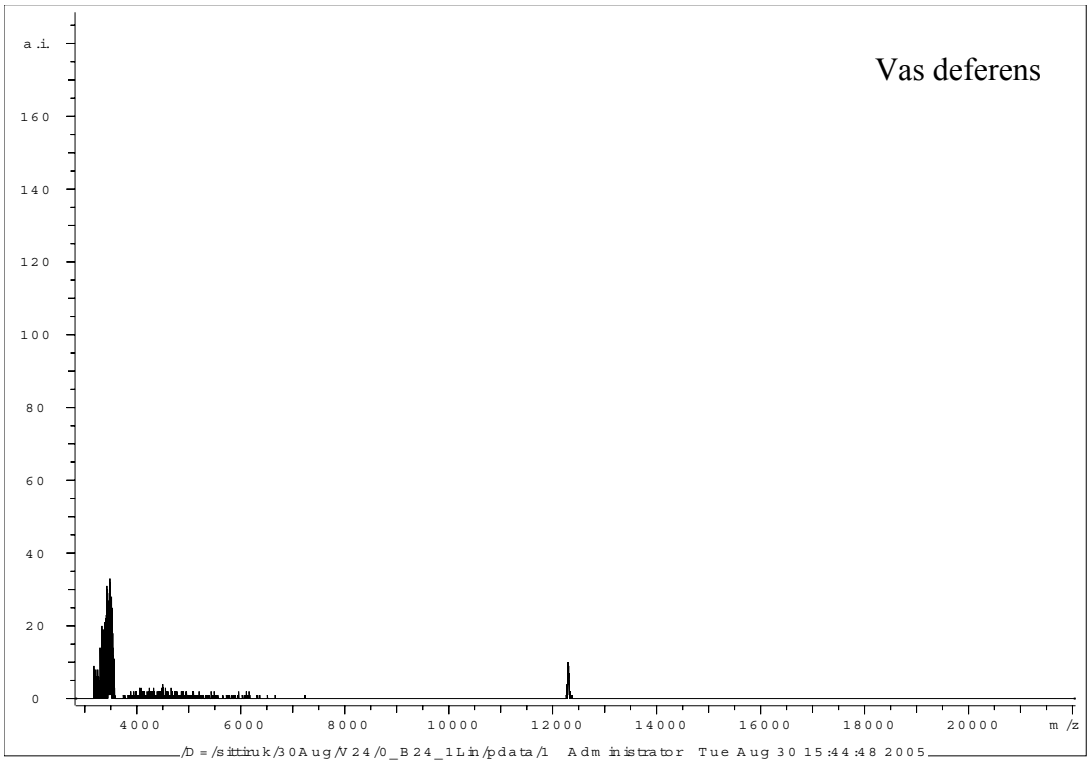


Appendix Figure 8 Fraction number 22 (eluted by 42.5% acetronitrile in 0.1%TFA)

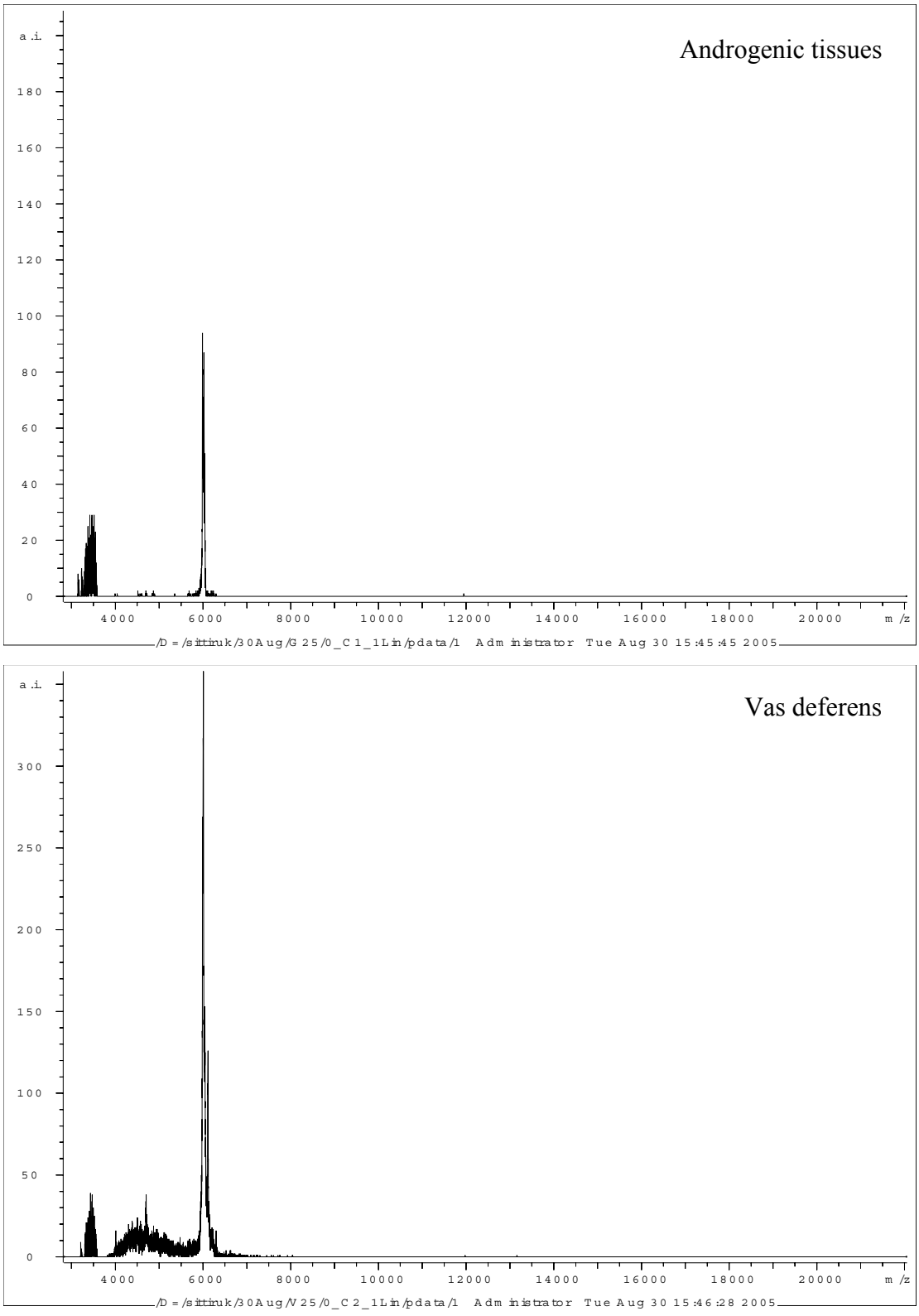


Appendix Figure 9 Fraction number 23 (eluted by 45% acetronitrile in 0.1%TFA)

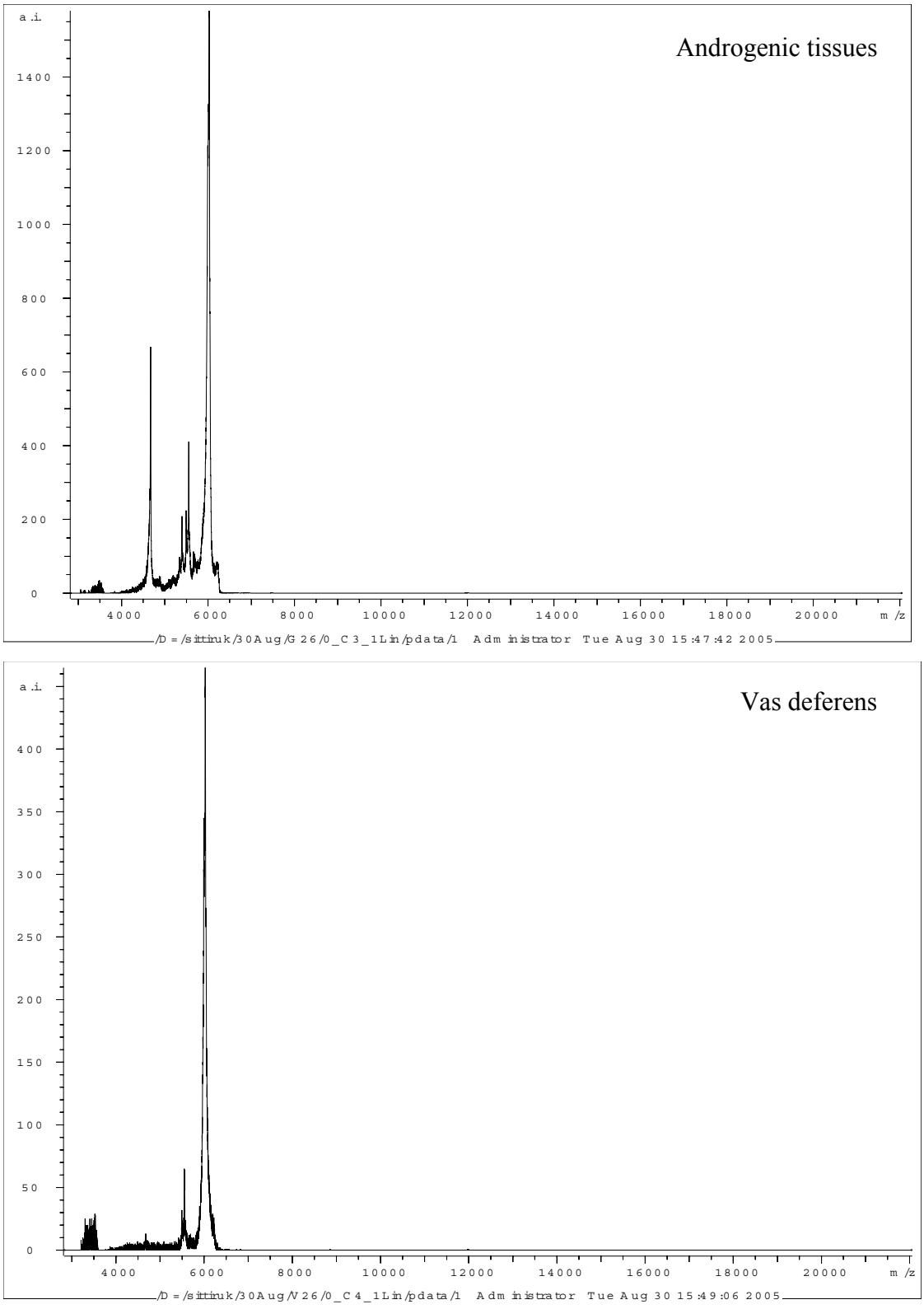




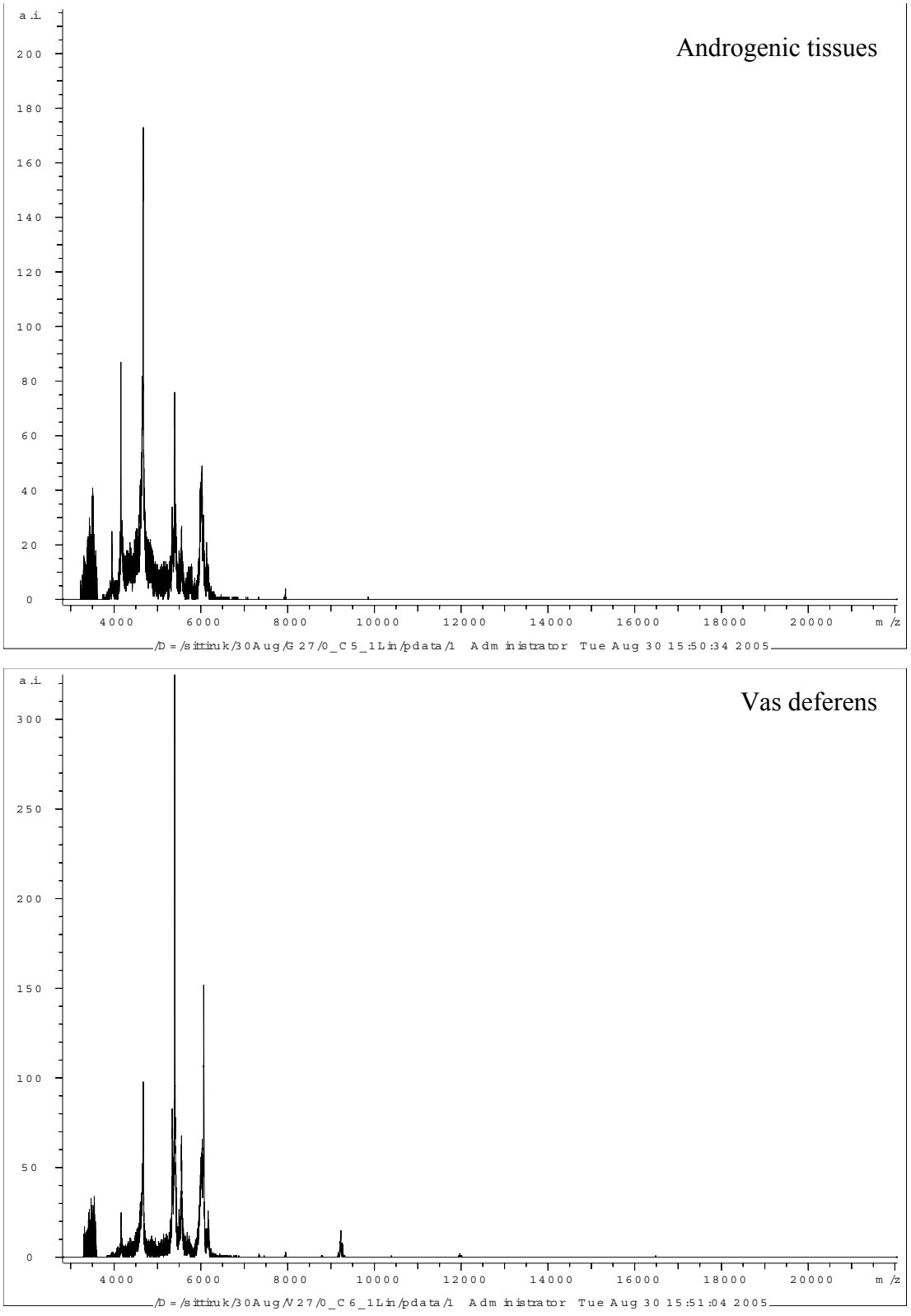
Appendix Figure 10 Fraction number 24 (eluted by 47.5% acetronitrile in 0.1%TFA)



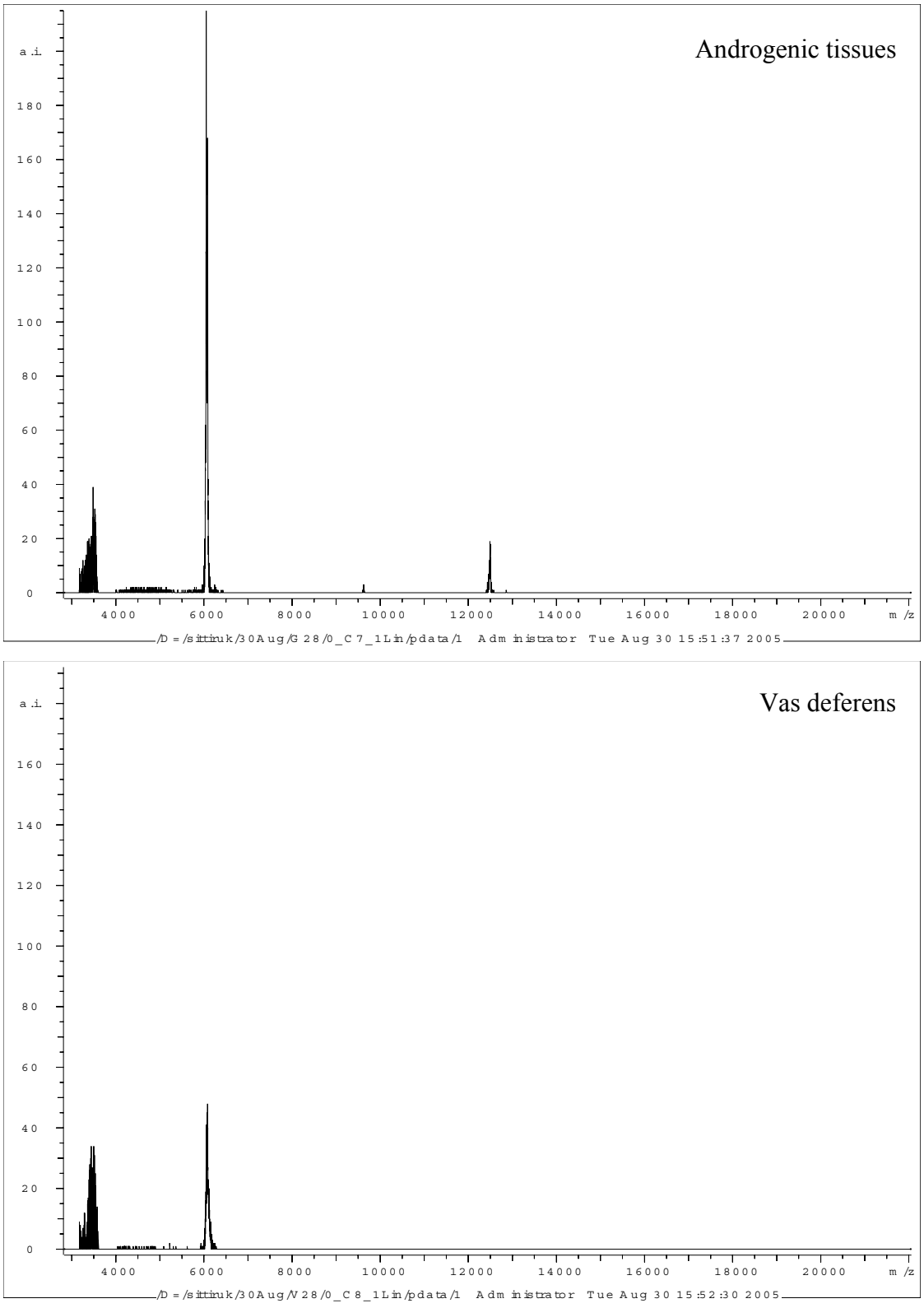
Appendix Figure 11 Fraction number 25 (eluted by 50% acetronitrile in 0.1%TFA)



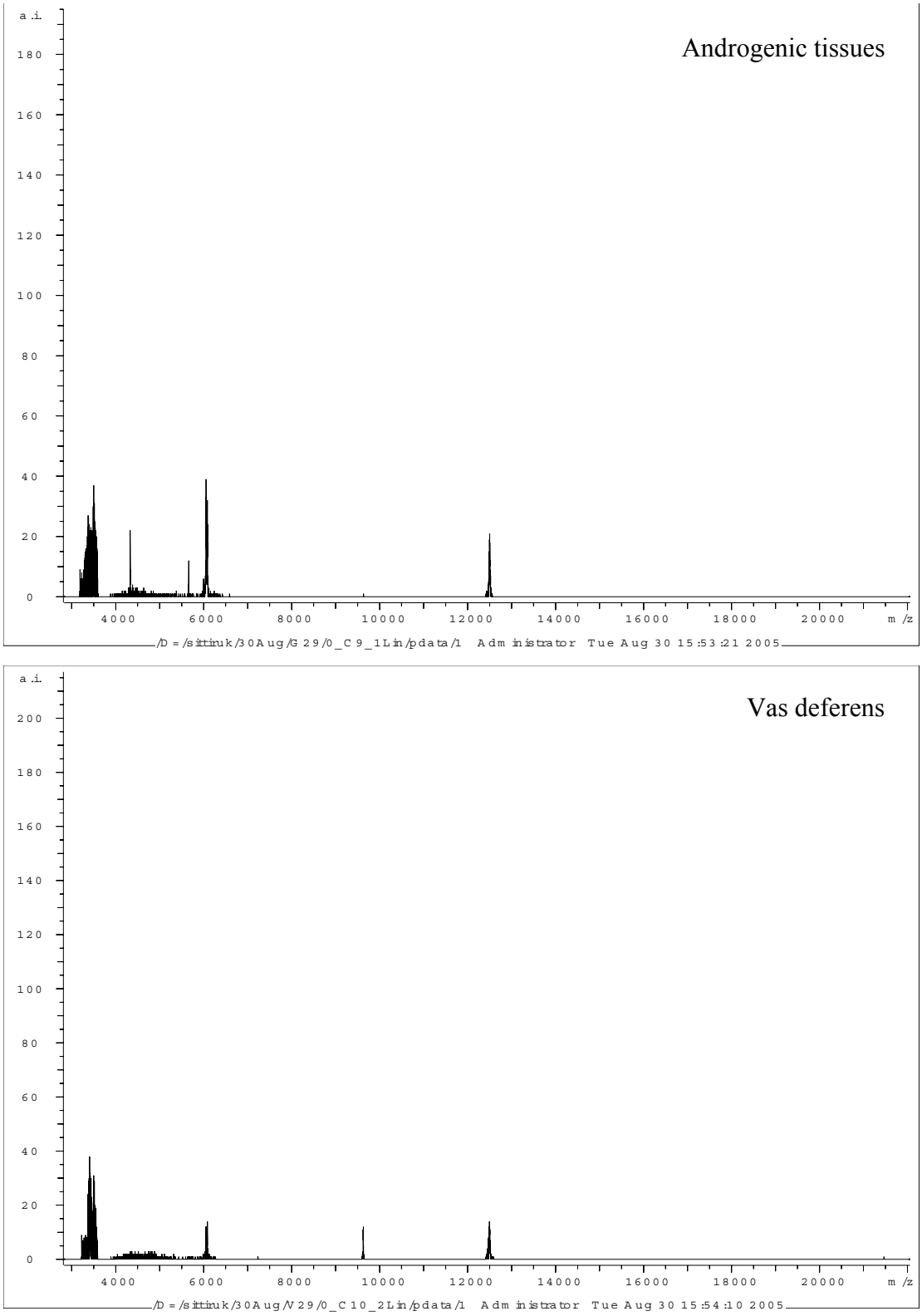
Appendix Figure 12 Fraction number 26 (eluted by 52.5% acetronitrile in 0.1%TFA)



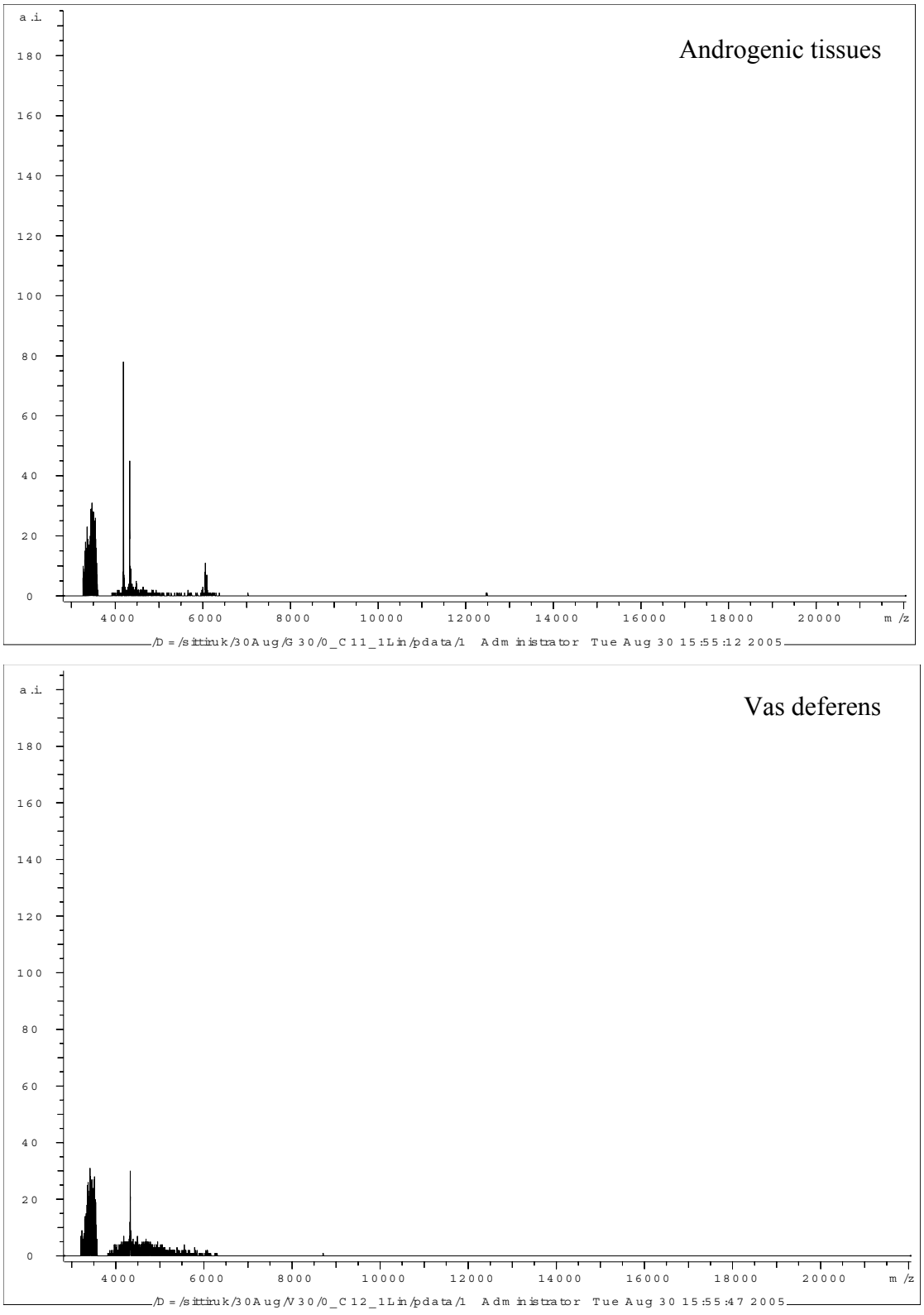
Appendix Figure 13 Fraction number 27 (eluted by 55% acetronitrile in 0.1%TFA)



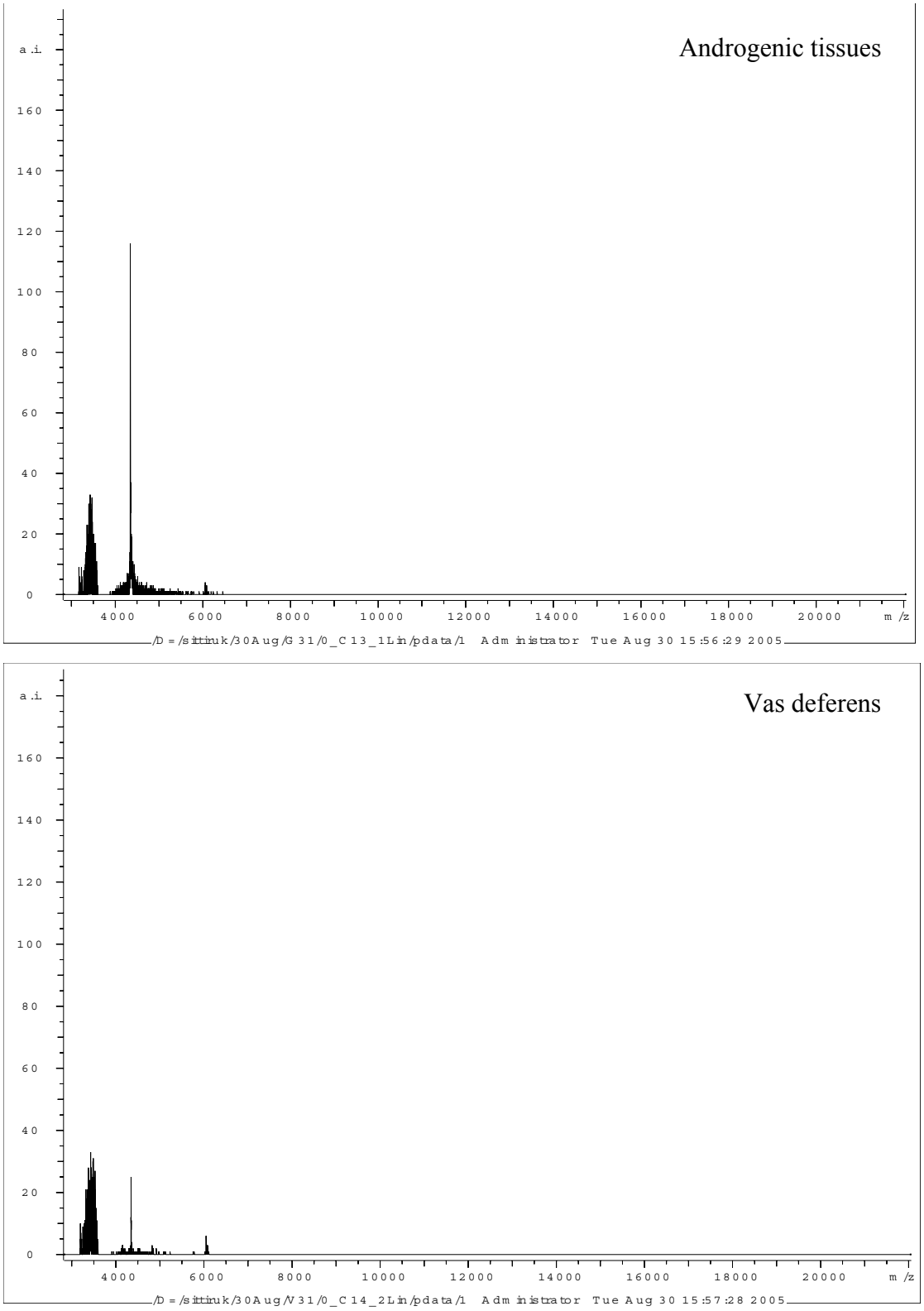
Appendix Figure 14 Fraction number 28 (eluted by 57.5% acetronitrile in 0.1%TFA)



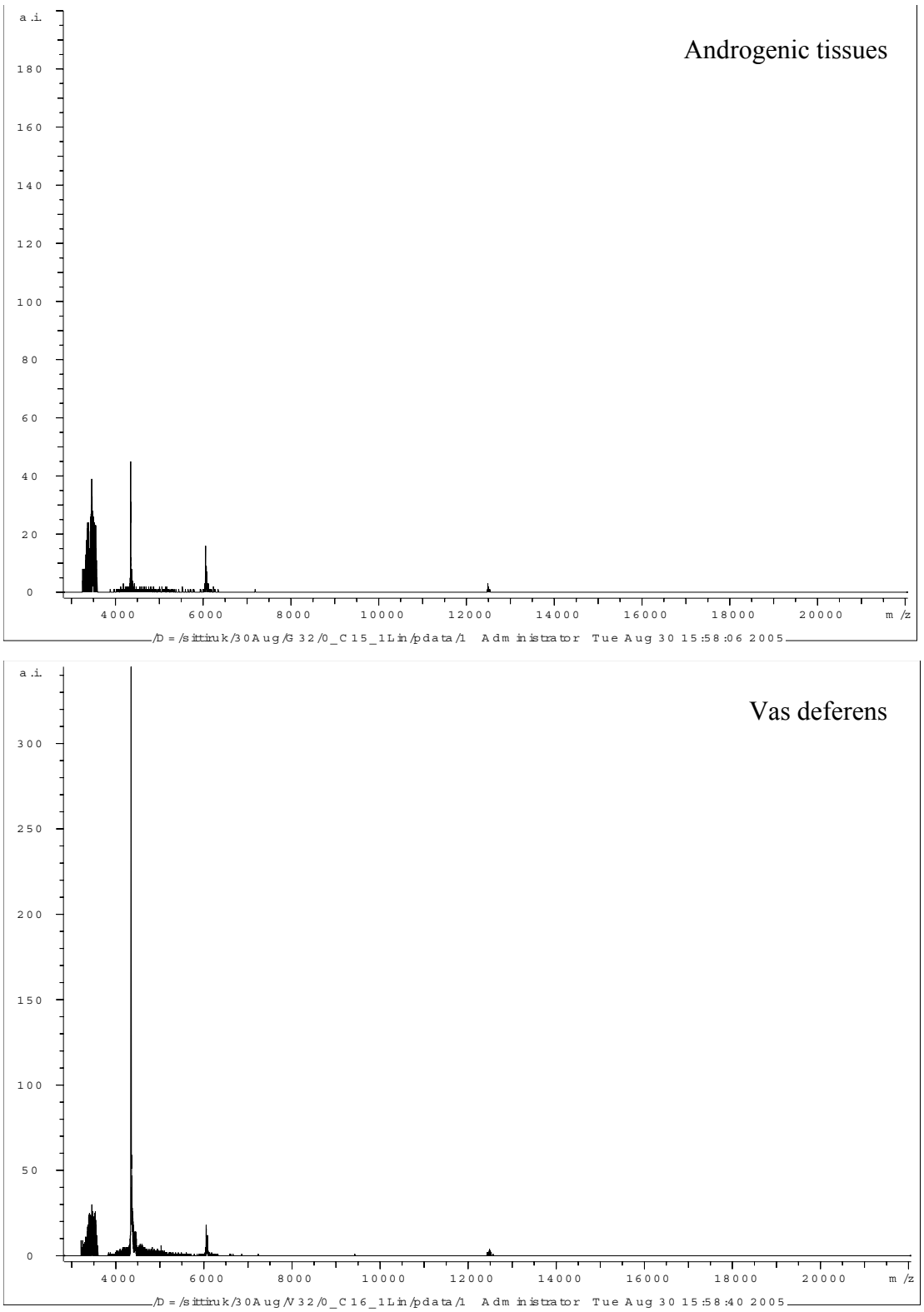
Appendix Figure 15 Fraction number 29 (eluted by 60% acetronitrile in 0.1%TFA)



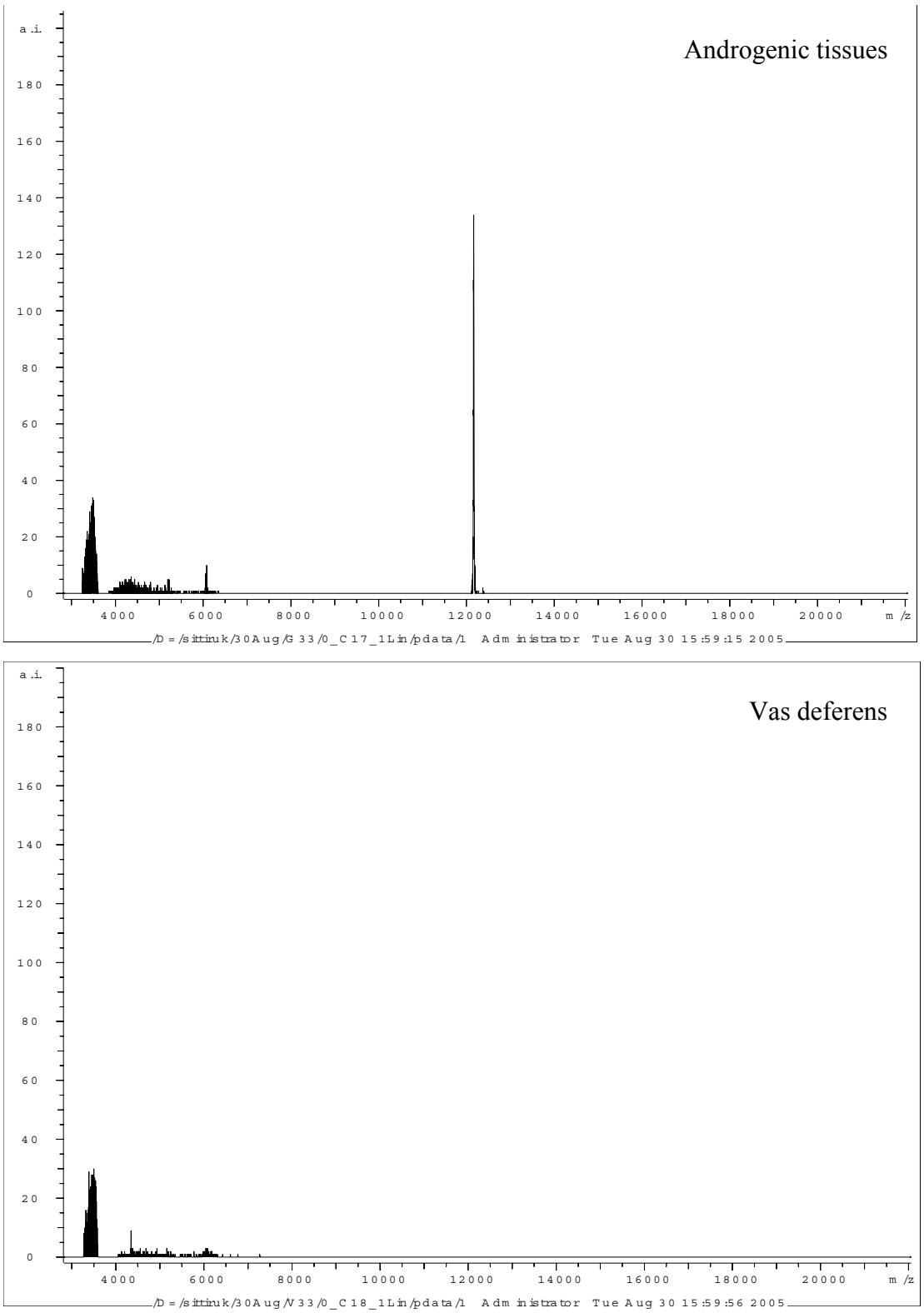
Appendix Figure 16 Fraction number 30 (eluted by 62.5% acetronitrile in 0.1%TFA)



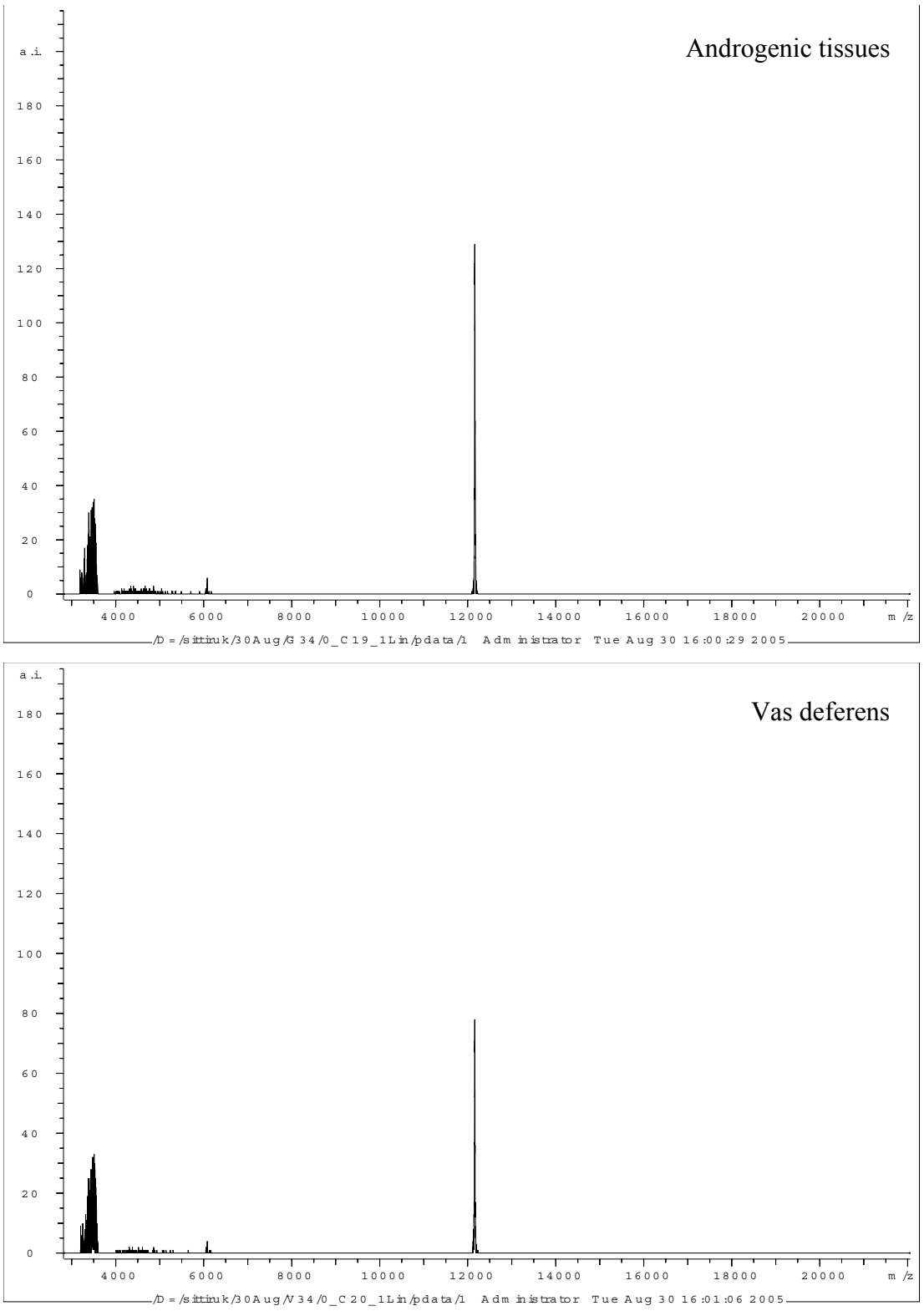
Appendix Figure 17 Fraction number 31 (eluted by 65% acetronitrile in 0.1%TFA)



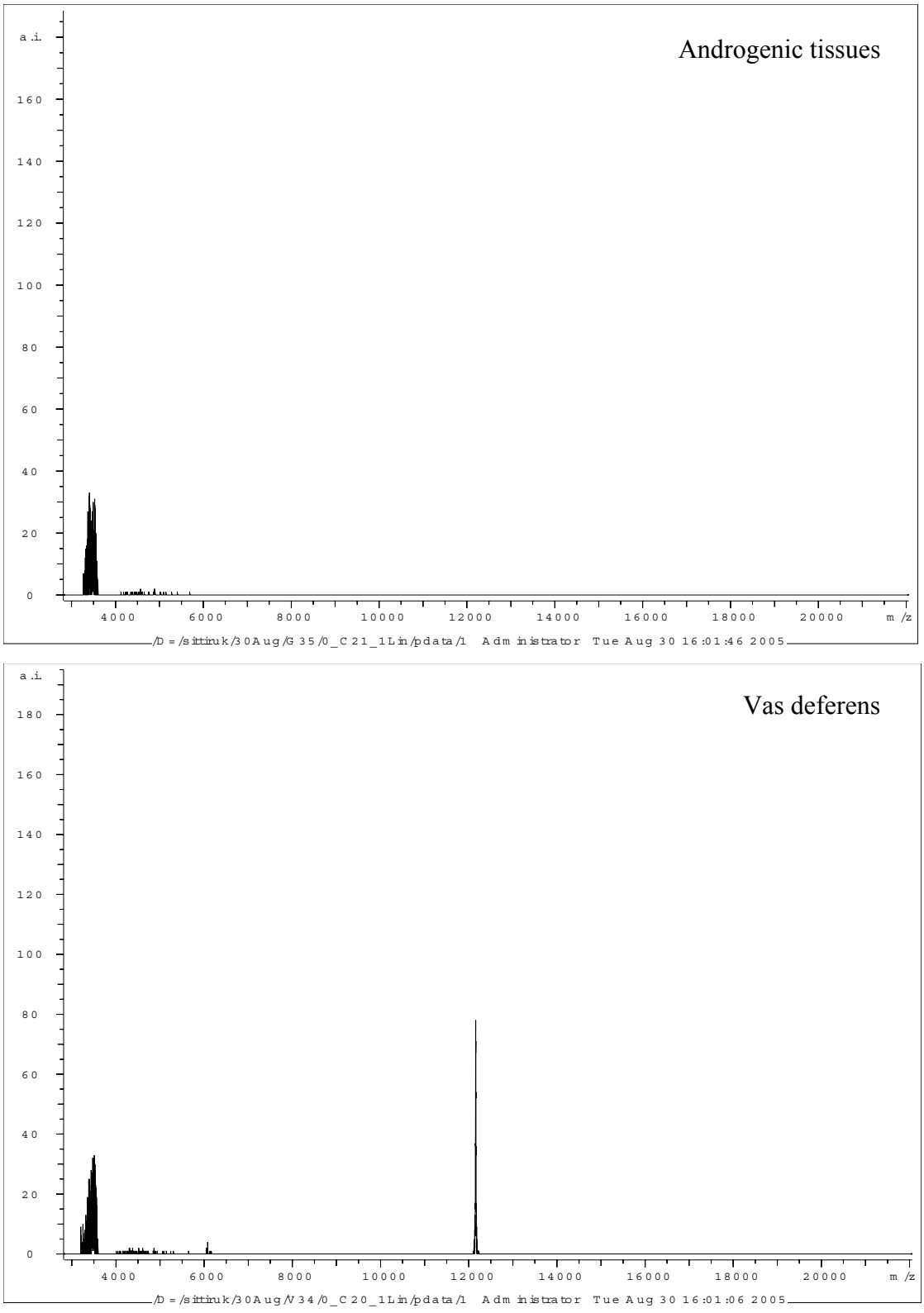
Appendix Figure 18 Fraction number 32 (eluted by 67.5% acetronitrile in 0.1%TFA)



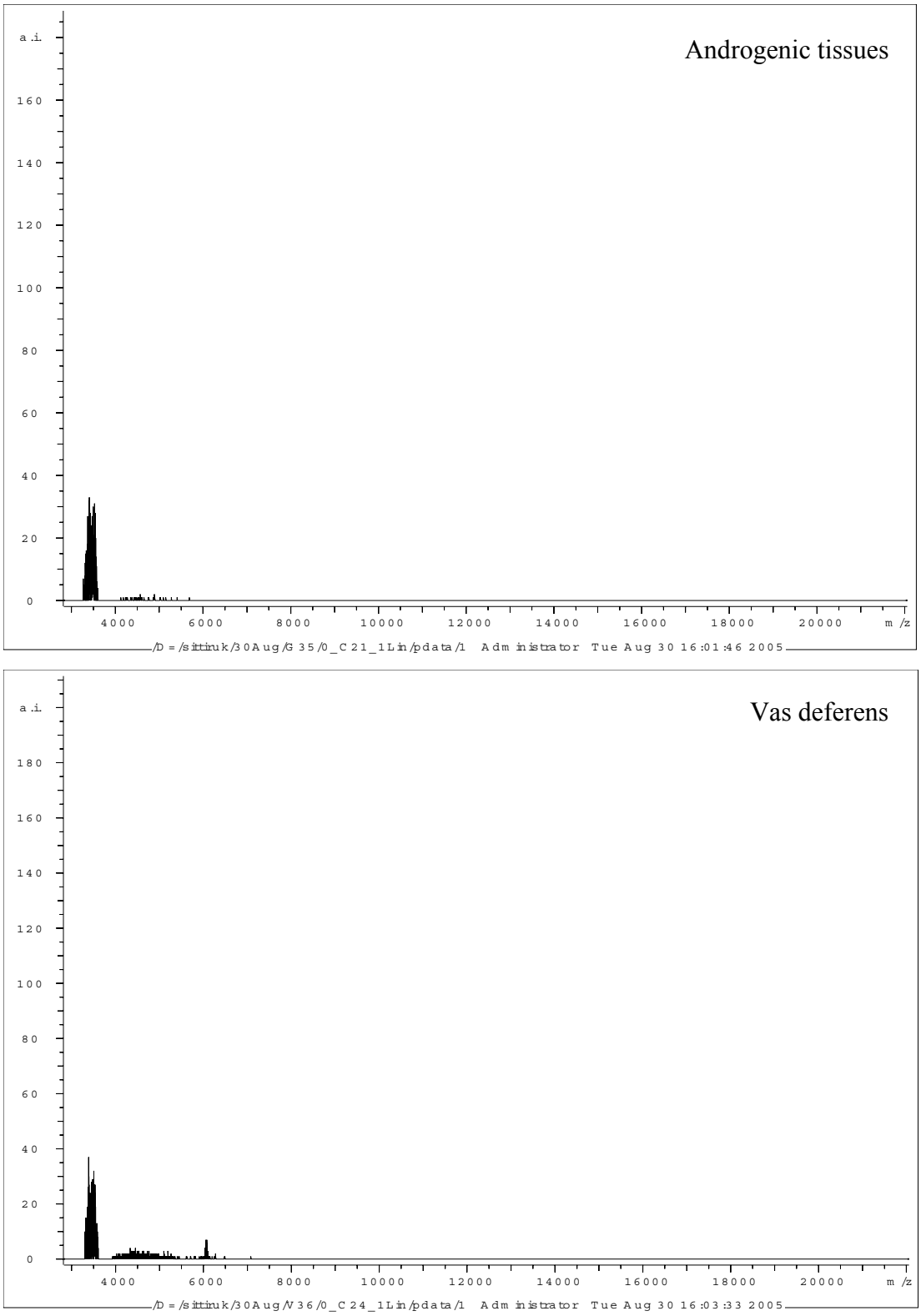
Appendix Figure 19 Fraction number 33 (eluted by 70% acetronitrile in 0.1%TFA)



Appendix Figure 20 Fraction number 34 (eluted by 72.5% acetronitrile in 0.1%TFA)



Appendix Figure 21 Fraction number 35 (eluted by 75% acetronitrile in 0.1%TFA)



Appendix Figure 22 Fraction number 35 (eluted by 77.5% acetronitrile in 0.1%TFA)

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