Asian Fisheries Science **18** (2005): 255-263
ISSN: 0116-6514
https://doi.org/10.33997/j.afs.2005.18.3.006
Asian Fisheries Society, Manila, Philippines

Accessory Nidamental Gland of *Sepia pharaonis* Ehrenberg (Mollusca:Cephalopoda): Ultrastructure and Function

J. RAJASEKHARAN NAIR, P. DEVIKA, M.C. GEORGE, M.J. SOPHIA and P.M. SHERIEF*

College of Fisheries ,
Kerala Agricultural University
Panangad P. O. Kochi- 682506
Kerala, India.

* *Corresponding author*

Abstract

The structure of the accessory nidamental gland (ANG) of the ripe female cuttlefish, *Sepia pharaonis* Ehrenberg has been investigated using transmission electron microscopy. The gland has certain structural features of a secretory organ containing a number of tubules filled with dense populations of coccoid and rod shaped bacteria. The wall of each tubule is lined by epithelial cells having structural specializations presumed to be involved in secretion. During sexual maturation of the cuttlefish, the ANG changes from colourless to mottled red due to the accumulation of carotenoid pigments in the bacterial population occupying the tubules. This indicates that the bacterial population responds to the sexual state of the host. Organic solvent extracts prepared from the ANGs exhibited antibacterial activity, but only during the final maturity phase of the animals. Thin layer chromatography (TLC) of the ANG extract showed the presence of lipid components such as phospholipids, cholesterol and free fatty acids. Thus, the ANG of *Sepia pharaonis* may be playing a secretory role in the reproductive cycle by coating the ovulated eggs with a layer of symbiotic bacteria having potent antibacterial property to ward off the egg pathogens.

Introduction

Squids and cuttlefish among decapod cephalopods, house symbiotic bacteria within their accessory nidamental gland (ANG). The ANG is a female accessory reproductive organ located anterior to the nidamental gland (NG) in squid and cuttlefish. The ANG-bacterial community association has been studied in loliginids (Bloodgood 1977, Biggs and Epel 1991, Lum-Kong and Hastings 1992, Barbieri et al. 1996) and in sepioids (van den Branden et al. 1979, 1980 and Grigioni et al. 2000). In the squid *Loligo pealei*, the ANG bacteria are transferred to the egg cases where they may have a protective antimicrobial function (Barbieri et al. 1997).

Sepia pharaonis (Pharaoh's cuttlefish) is a commercially important species distributed in the Indo-Pacific geographical area. The ripe female of *S. pharaonis* has a large nidamental gland complex (NGC) with a prominent ANG of mottled red colour. The ANG of the immature and maturing females lack the mottlings, which appear only when the females are ripe and ready to spawn. The present study investigates the ultrastructure of the ANG and the antibacterial activity of the ANG extract. Attempts are also made to understand the biochemical nature of the extract by spectral analysis, TLC separation and a study of thermal stability.

Material and Methods

Test animals

Twelve *S. pharaonis* ripe females were collected from the Kochi fishing harbour (south-west India) during March 2002 coinciding with the peak spawning period (Silas et al. 1982). The specimens were transported in insulated box with ice to the laboratory. The total mantle length and total weight were measured. The animals were dissected asceptically to collect the NGC. The NG and ANG were weighed to compute the somatic indices.

Electron microscopy

The isolated ANG was fixed in 3% cacodylate buffered glutaraldehyde (0.1 M) for 1 h at 4°C. The gland was trimmed to 1 mm^3 pieces using a razor blade and kept in fresh glutaraldehyde for 3 h. The pieces were given three washes in cacodylate buffer for 30 min each. The pieces were kept in buffer overnight and then post-fixed in 1% osmium tetroxide in buffer for 1 h at 4°C , dehydrated in a graded series of acetone-water mixtures and embedded in Spurr's Low Viscosity Embedding Medium (Spurr 1969).The tissue was sectioned with an LKB ultramicrotome, stained in

uramyl acetate and lead citrate and photographed using a Hitachi H-600 transmission electron microscope.

Antibacterial activity

The ANG was extracted with different solvent systems: water, acetone:alcohol (7:2 v/v), ethanol, methanol and butanol at the rate of 3.5 g tissue per 10 ml solvent. The extract was centrifuged at 10000 rpm for 20 min at 4°C in a biofuge. The clear supernatant was used for the antibacterial assay by the standard disc diffusion method using 6 mm disc. Each disc was impregnated with 250 µl of ANG extract and vacuum dried. The activity was measured as diameter of the inhibition zone in mm. The bacterial strains used for the assay were *Escherichia coli*, *Staphylococcus aureus*, *Aeromonas hydrophila* and *Bacillus megaterium*. The thermal stability of the antibacterial agent in the butanol ANG extract was tested at 40-100ºC at 10ºC intervals. For this blank discs were impregnated with 250 µl of the extract and incubated for 30 min at respective temperatures. The discs were then vacuum dried and the antibacterial activity was assayed against *E. coli* and *A. hydrophila*.

Spectral analysis

Pigment was extracted from ANG using a 7:2 (v/v) mixture of acetone: ethanol (Bloodgood 1977). After overnight incubation at room temperature, the extract was centrifuged at 10000 rpm for 20 min in a biofuge at 4°C. The supernatant was filter-sterilized and the absorbance spectrum of the pigment extract was measured in a spectrophotometer (Jasco, Japan) at 200-600 nm. The absorbance spectrum of the butanol extract was also measured for comparison. The dry matter content of the extracts were estimated gravimetrically.

Thin layer chromatography

The butanol extract of the ANG was tested for polyphenols/ flavanoids by using 1% alcoholic ferric chloride as spray reagent after TLC on silica gel G with n butanol:acetic acid:water (4:1:5 v/v/v) as solvent system. Quinones were tested using 10% alcoholic potassium hydroxide as spray reagent. TLC was also carried out for the separation and identification of the lipid components in the ANG butanol extract with hexane:ether:acetic acid (85:15:2 v/v/v) as the solvent system. The plates after drying were sprayed with 50% sulphuric acid and the separated lipid components were identified using standards.

Results

The total mantle length ranged from 15.8 – 24.0 cm and the total body weight from 450.0 – 1400.0 g for the 12 ripe females. The NGC-somatic index was 7.3 ± 1.24, the NG-somatic index was 5.85 ± 1.16 and the ANG-somatic index was 1.26 ± 0.17. The accessory nidamental gland is attached to the anterior aspect of the nidamental gland and the complex is closely adhering to the dorsal surface of the ink duct (Fig. 1).

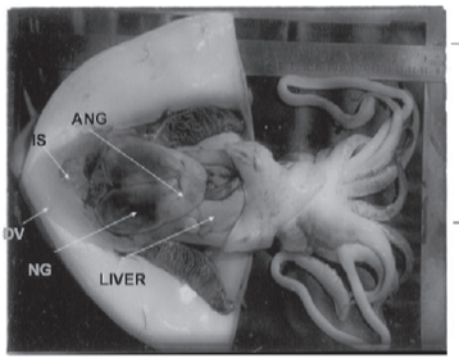

Fig. 1. The viscera of *S. pharaonis* showing the relative position of the nidamental gland complex. (NG- nidamental gland, ANG-accessory nidamental gland, IS- ink sac, OV- ovary).

Transmission electron micrograph (TEM) of the ANG showed epithelial cell lining the tubule harbouring the bacterial population (Fig. 2). Dividing bacterium was observed within the ANG tubule (Fig. 3). The epical or lumenal surface of the epithelial cell contains short microvilli, cilia and globular bodies (Fig. 4), suggesting a secretory role for the ANG.

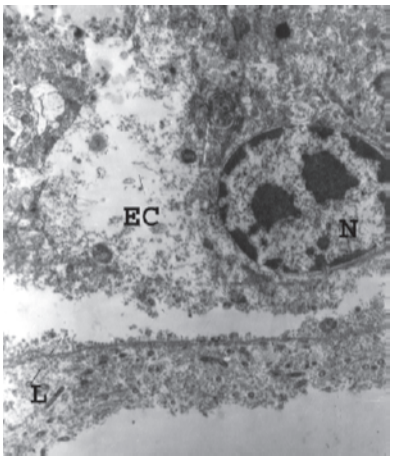

Fig. 2. Transmission electron micrograph (TEM) of the ANG showing the epithelial cell lining the tubule containing bacterial population. (EC-epithelial cell, N-nucleus, L-lumen). x 6000.

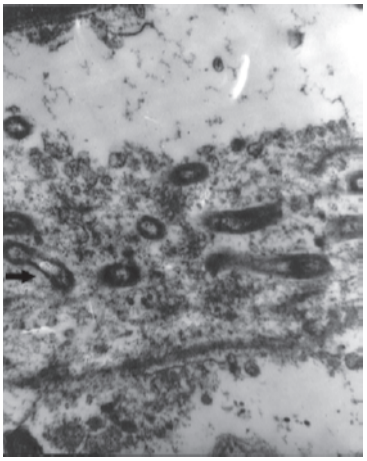

Fig. 3. High power TEM of the ANG tubule showing a dividing bacterium (arrow mark). x 30,000.

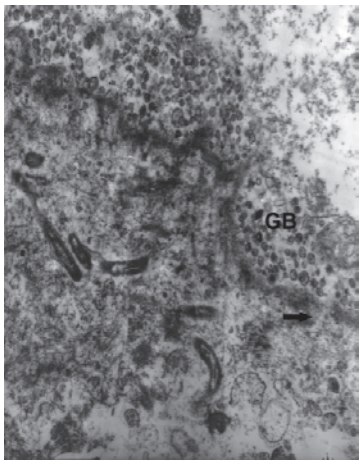

Fig. 4. High power TEM of the ANG showing the lumenal surface of the epithelial cell containing secretory globular bodies (GB). The arrowhead points to the opening at the secretory site into the tubule lumen. x 25,000.

During the antibacterial assay only the organic solvent extracts of the ANG exhibited antibacterial activity (Table 1).Of the different organic solvents tried butanol extract showed the highest activity followed by methanol extract. The extracts were active against both gram positive and gram negative organisms, the inhibition being more against gram negative bacteria. Table 2 shows the antibacterial activity of the

butanol extract at different temperatures (40- 100°C) at 10°C intervals. The results showed that the bioactive agent present in the extract is thermally stable.

The absorption peaks for the acetone: alcohol extract and butanol extract showed two major absorption peaks at 339.5 and 453.0 nm for the acetone: alcohol extract and three peaks at 285.0, 339.5 and 453.0 nm for the butanol extract (Table 3). The peak at 453 nm can be attributed to the presence of carotenoid pigments. The peak obtained at 285 nm for the butanol extract indicates the presence of peptides/ proteins. The dry matter content of acetone: alcohol extract was 5.46% and for the butanol extract 6.46%.

The TLC of the butanol extract did not show the presence of any polyphenols/flavanoids and quinones whereas lipid components such as phopholipids, cholesterol and free fatty acids were detected (Fig. 5).

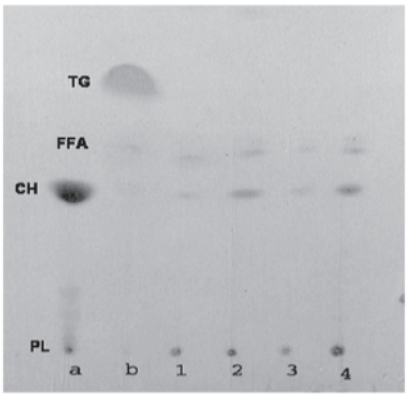

Fig. 5. Thin layer chromatography of the ANG-butanol extract showing the lipid components.(Lane a : Mixture of phospholipid (PL) and cholesterol (Ch) standards. Lane b : Mixture of free fatty acid (FFA) and triglyceride (TG) standards. Lanes 1,2,3 & 4 : Sample replicates).

Table 1. Antibacterial activity of the organic solvent extracts of ANG of *S. pharaonis*

Extract	Bacterial Strain			
	E.coli	***A. hydrophila***	***S. aureus***	***B. megaterium***
Acetone: alcohol (7:2 v/v)	8.4 mm	8.1 mm	7.4 mm	7.1 mm
Ethanol	6.5 mm	6.4 mm	6.3 mm	NA
Methanol	9.4 mm	8.3 mm	7.9 mm	7.9 mm
Butanol	10.1 mm	9.3 mm	8.3 mm	8.0 mm

Values are mean of four replicates.
NA- No activity.

Table 2. Antibacterial activity of ANG-butanol extract at different temperatures

Bacterial strain	Temperature						
	40°C	**50°C**	**60°C**	**70°C**	**80°C**	**90°C**	**100°C**
A.hydrophila	7.75 mm	8.5 mm	8.0 mm	7.75 mm	9.0 mm	8.5 mm	9.0 mm
E. coli	8.0 mm	7.25 mm	8.0 mm	7.0 mm	8.0 mm	9.0 mm	9.0 mm

Values are mean of two replicates.

Table 3. λ max for different peaks of absorbance of pigments in ANG extracts

Extract	λ max		
	1	2	3
Acetone:alcohol (7:2 v/v)	-	339.5 (0.266 A)	453.0 (0.414 A)
Butanol	285.0 (2.291 A)	339.5 (0.350 A)	453.0 (0.529 A)

Values in brackets indicate the respective absorbance.

Discussion

The ultrastructure of the ANG of *S. pharaonis* suggests the presence of a number of tubules in the gland with each tubule lined by a layer of epithelial cells. The cilia present on the lumenal surface of the epithelial cell may have a co-ordinated mobility providing a flow of material along the tubule lumen. The lumenal surface of the tubule reveals, besides microvilli and cilia, specializations that may act as sites of secretion. Innumerable number of globules seen at these secretory sites are interpreted as material in the process of being secreted through the opening into the tubule lumen. The EM studies, thus, suggest secretory role of the ANG. Bloodgood (1977), who studied the ultrastructure of the squid (*Loligo pealei*) ANG has pointed out that the gland has many of the structural features of a secretory organ. Kaufman et al. (1998) reported that the ANG of *L. opalascens* displayed tubules composed of a single layer of epithelial cells and expressing numerous cilia and microvilli.

The electron microscopic studies also reveal a mixed population of coccoid and rod shaped bacteria in the lumen of the ANG tubules of *S. pharaonis* ripe females. Bloodgood (1977) revealed the presence of a suspension of coccoid and rod-shaped bacteria in the ANG tubules of *L. pealei*. In *S. officianalis* coccoid and rod shaped bacteria, Gram positive or variable, occurred together in the ANG tubules (Grigioni et al. 2000). They also identified the bacterial groups as *Roseobacter*, *Agrobacterium*, *Sporichthya*, *Rhodobium-Xanthobacter* and *Clostridium* using molecular techniques. Barbieri et al. (1997) attributed an antibacterial function to *Alteromonas* and *Shewanella*, ANG symbionts of *L. pealei*. These bacteria were not detected in *S. officianalis* but Grigioni et al. (2000) states "antimicrobial activity by other strains present in *Sepia* cannot be excluded". In *S. pharaonis* the ANG harbours a dense bacterial population and the extract exhibits broad-spectrum antibacterial activity. The bacterial strains need further characterization and antimicrobial evaluation. Sherief et al. (2004) have also reported antibacterial activity in the ANG-butanol extracts of *Sepia aculeata*, *Sepiella inermis* and *Loligo duvaucelii*.

In *S. pharaonis* the ANG seem to respond to the sexual state since they change from colourless in immature females to mottled- red in mature females. Symbiotic bacteria are known to interact with the host. Bloodgood (1977) reported the presence of three types of tubules – red, white and yellow, the tubules of similar colour tend to be clustered together giving the gland a mottled appearance in *L. pealei*, as also seen in the present study. In *S. pharaonis* the absorption peaks for the pigments in the ANG extracts show the accumulation of carotenoids. Van den Branden et al. (1979) has characterized the most important pigment – the orange-red xanthophyll called sepiaxanthin in *S. officianalis*. Bacteria belonging to *Roseobacter* and *Rhodobium – Xanthobacter* are phototropic and could be responsible for the red- orange colour of the ANG in the mature *S. officianalis* females due to carotenoid accumulation (Grigioni et al. 2000).

Bloodgood (1977) suggested that putative sexual hormones affect the functioning of the ANG and that material secreted by ANG epithelial cells may alter the metabolism of the bacteria. As cited by Arnold (1984), Richard and co-workers in the 1960s have done a very thorough study of the ovarian maturation in the European cuttlefish *S. officianalis* by using the colour and size of ANG as maturity indices. They have shown that the optic gland gonadotropin controls gonad maturation. Thus short days stimulate maturation through the optic gland gonadotropin and long days and higher temperatures of the shallow waters "stimulate" egg laying. In the case of *S. pharaonis* the appearance of mottled-red colouration of the ANG did not guarantee antibacterial activity. Only the ANG of sexually ready to spawn females with ovulated eggs exhibited antibacterial activity, supporting the role of a putative hormone in ANG metabolism. Ram (1977) demonstrated the presence of an egg laying hormone (ELH) in opisthobranchs inducing ovulation, egg transport, fertilization and packaging in the egg string and its fixation to the substrata.

As early as 1918, observing a bacterial coating of the eggs in *Sepia officianalis*, Pierantoni (1918) deducted a possible vertical transmission of ANG bacteria from generation to generation. During the collection of a large number of specimens for the present project, it was observed that the NGC was virtually absent in the sexually undifferentiated immature individuals and the gland complex develops during maturation. The tubules of the ANG are open to the mantle cavity and to seawater, possibly helping the cuttlefish to acquire its bacterial symbionts horizontally from the ambient medium. Such a postulate was also put forth by Kaufman et al. (1998) who observed that the ANG organization took place only at 129 days post-hatching in *L. opalascens* life history. Lum-Kong and Hastings (1992) also report on the possibility of acquiring of the symbionts (species of *Vibrio* and *Pseudomonas*) from the surrounding environment after hatching in *L. forbesi*.

Benkendorff et al. (2001) suggest that the egg masses of marine molluscs appear to have broad spectrum antimicrobial activity with the gram-positive bacteria

showing more susceptibility. The butanol extract of the ANG in *S. pharaonis* showed broad spectrum antibiotic activity with the gram-negative bacteria being more susceptible. Kurihara et al. (1999) report on the antibacterial activity of free fatty acids obtained from dried sea weed *Gloiopeltis furcata*. The free fatty acids detected in the ANG extracts of *S. pharaonis* require further investigation.

The present study confirms the findings of Bloodgood (1977) and van den Branden et al. (1979) that the ANG is a secretory organ made up of a number of tubules harbouring a dense symbiotic bacterial population. As suggested by van den Branden et al. (1979) in the case of *S. officianalis*, Biggs and Epel (1991) in the case of *L. opalascens* and Barbieri et al. (1997) in the case of *L. pealei*, the ANG of *S. pharaonis* may be playing a secretory function in the reproductive cycle for coating the ready to be fertilized ovulated eggs with a layer of symbiotic bacteria having potent antibacterial property to protect the developing eggs from pathogens.

Acknowledgment

The financial support of the N A T P of the Indian Council of Agricultural Research, New Delhi and of the D B T, Govt. of India is gratefully acknowledged. We also thank the Dean, College of Fisheries, Panangad for the facilities extended. The authors are also grateful to Dr. N.K. Sanil, CMFRI, Kochi for his technical help in electron microscopy.

References

Arnold, J M. 1984. Cephalopods. In: The Mollusca. Vol VII. Reproduction (eds. A.S. Tompa, N.H. Verndonk and van den Biggelaar), pp. 419-454. Academic Press Inc., Florida.

Barbieri, E., J. Gulledge, D. Moser and C. Chien. 1996. New evidence for bacterial diversity in the accessory nidamental gland of the squid (*Loligo pealei*). Biological Bulletin 191: 316-317.

Barbieri, E., K. Barry, A. Child and N. Wainwright. 1997. Antimicrobial activity in the microbial community of the accessory nidamental gland and egg cases of *Loligo pealei* (Cephalopoda: Loliginidae). Biological Bulletin 193(2):275-276.

Benkendorff, K., A.R. Davis and J.B. Bremner. 2001. Chemical defense in the egg masses of benthic invertebrates:An assessment of antibacterial activity in 39 molluscs and 4 polychaetes. Journal of Invertebrate Pathology 78:109- 118.

Biggs, J. and D. Epel. 1991. Egg capsule sheath of *L. opalascens* Berry : Structure and association with bacteria. Journal of Experimental Zoology 259:263-267.

Bloodgood, R.A. 1977. The squid accessory nidamental gland: Ultrastructure and association with bacteria. Tissue & Cell 9(2): 197-208.

Grigioni, S., R. Boucher-Rodoni, A. Demarti, M. Tonolla and R. Peduzzi. 2000. Phylogenetic characterization of bacterial symbionts in the accessory nidamental glands of the sepioid *Sepia officianalis* (Cephalopoda:Decapoda). Marine Biology 136: 217-222.

Kaufman, M.R., Y. Ikeda, C. Patton, G. Van Dykhuizen and D. Epel. 1998. Bacterial symbionts colonise the accessory nidamental gland of the squid *Loligo opalascens* via horizontal transmission. Biological Bulletin 194(1): 36-43.

Kurihara, H.,Y. Goto, M. Aida, M. Hosokawa and K. Takahashi. 1999. Antibacterial activity against cariogenic bacteria and inhibition of insoluble glucan production by free fatty acids obtained from dried *Gloiopeltis furcata*. Fisheries Science 65(1): 129-132.

Lum-Kong, A. and T.S. Hastings. 1992. The accessory nidamental glands of *Loligo forbesi* (Cephalopoda: Loliginidae): characterization of symbiotic bacteria and preliminary experiments to investigate factors controlling sexual maturation. Journal of Zoology, London 228: 395-403.

Pierantoni, A. 1918. Organi luminosi, organi simbiotici e glandola nidamentale accessoria nei cefalopodi. Bolleti Societi Naturale Napoli 30:30-36.

Ram, J.L. 1977. Hormonal control of reproduction in *Busicon*: Laying of egg capsules caused by the nervous system extracts. Biological Bulletin 152: 221-232.

Sherief, P.M., M.C. George, J.R. Nair, P. Devika, M.J. Sophia and Priya, S.V. 2004. Antibacterial activity in the extract of accessory nidamental glands of squid and cuttlefish. Proceedings of MBR 2004 National Seminar on New Frontiers in Marine Bioscience Research pp.47-51. National Institute of Ocean Technology, Chennai.

Silas, E.G., K.S. Rao, R. Sarvesan, K.P. Nair and M.M. Meiyappan. 1982. The exploited squid and cuttlefish resources of India: A review. Marine Fisheries Information Service Technical and Extension Series No.34:1-16.

Spurr, A.R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. Journal of Ultrastructure Research 26:31-43.

Van den Branden,C., A. Richard, J. Lemaire and W. Decleir. 1979. La glande nidamentaire accessoire de *Sepia officianalis* L.: analyses biochemiques des pigments des bacteries symbiotiques. Annales de Societe Zoologique, Belgium 108:123-139.

Van den Branden, C., M. Gillis and A. Richard. 1980. Carotenoid producing bacteria in the accessory nidamental glands of *Sepia officianalis*. Comparative Biochemistry and Physiology 66(B): 331-334.