Antibacterial activity in the accessory nidamental gland extracts of the Indian squid, *Loligo duvauceli* Orbigny

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Antibacterial activity of accessory nidamental gland-butanol extracts from the Indian squid *Loligo duvauceli* in different stages of maturity was studied. The activity was evaluated by disc-diffusion method using five strains of bacteria. The extracts from ripe stage ANG showed antibacterial activity against gram negative bacterial strains, *Escherichia coli* and *Pseudomonas aeruginosa*, and gram positive bacteria, *Staphylococcus aureus*. Immature and spent gland extracts did not show any antibacterial activity. Spectrophotometric analysis of the ripe gland extract showed the maximum absorbance at 498.5 nm. This infers the presence of carotenoid pigments which impart the orange red colour to the ripe glands. Thin layer chromatography of the ANG-butanol extract revealed the presence of lipid components such as phospholipids, cholesterol, free fatty acids, triglycerides, fatty acid esters, and cholesteryl esters. The total free fatty acid content was significantly higher in the ripe ANG (16.0 ± 0.143 mg oleic acid/g tissue), in comparison to the immature ANG (10.3 ± 0.114 mg oleic acid/g tissue). Gas chromatographic studies of immature and ripe stages revealed the presence of a mixture of fatty acids. The major unsaturated fatty acids content in the ripe stage was 1.973 mg/gm tissue, whereas in immature stage it was only 0.251 mg/gm tissue. Significantly higher levels of unsaturated fatty acids in the ripe stage could be the factor responsible for the antibacterial activity of the ANG-butanol extract.

(Key words: squid, accessory nidamental glands, antibacterial activity, chromatography, fatty acids)

Introduction

Natural bioactive substances have the least quantum of side effects when compared to synthetic products. Although most antibiotics have been derived from terrestrial life, it is the marine world that may provide the pharmaceutical industry with the next generation of medicines. The biochemistries of seemingly simple marine organisms such as blue green algae, sponges and squid are inspiring new ideas for drug development.

Accessory nidamental glands (ANG) are present in the sexually mature females of cephalopods. These paired glands are located at the anterior end of the nidamental glands and are closely associated with the ventral surface of ink sac (Fig. 1). These glands are reported to harbour dense bacterial community and the ANG-bacterial community association has been studied in loliginids and sepioids. Because of its close association with the nidamental glands and the oviduct, the ANG has long been thought to play a role in protecting the eggs by coating them with symbiotic bacteria or bacteriosins to ward off pathogens or predators. The antibacterial properties of the ANG extracts from *L. pealei* and from *Sepia aculeata, S. pharaonis* and *Sepiella inermis* have been reported.

Materials and Methods

Females of the Indian squid, *Loligo duvauceli* in different stages of maturity (immature, ripe and spent stages) were collected from the fish processing plant, Bhatson’s Aquatic Products, Aroor, Alleppey District, Kerala, during the peak period of maturity (September-November, 2007). The animals were brought to the laboratory in ice cold condition. The accessory nidamental glands were dissected out and removed aseptically. The color and weight of the ANGs were noted. The glands were kept frozen at -20°C till further use. Bacterial strains were obtained from National Institute of Oceanography, Regional Centre, Kochi. All the strains were maintained on nutrient agar.

Exhaustive method of extraction with butanol was used with 2.5 g ANG in 5.0 ml butanol. The supernatant of the extract was filter sterilized. The...
extract was stored at -20°C for the antibacterial assay and spectrophotometric, thin layer chromatographic (TLC) and gas liquid chromatographic (GLC) analyses.

The antibacterial activity was tested by filter paper disc diffusion technique. Filter paper discs of 6 mm size (Whatman No.1 filter paper) were impregnated with 250 µl of ANG extract and the discs were vacuum dried. The activity was measured as the diameter of the inhibition zone in mm. Standard chloramphenicol discs and butanol impregnated discs were used as the positive and negative controls respectively.

The absorbance spectra of the ANG-butanol extracts were measured on a spectrophotometer (Jasco, Japan) at 200–600 nm.

The butanol extract of the ANG in different maturity stages were further tested for polyphenols/flavonoids by using 1% alcoholic ferric chloride as a spray agent after TLC on silica gel G with butanol: acetic acid:water (4:1:5v/v/v) as a solvent system. Quinones were tested using 10% alcoholic potassium hydroxide as a spray agent. 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.

The free fatty acid (FFA) content of the immature and ripe stage ANG extract was also determined. For this, a known volume of extract was evaporated to dryness and re-dissolved in neutralized 95% ethanol. This was titrated against 0.01 N NaOH solution using phenolphthalein as an indicator. End point was formation of pink color that persisted for 30 seconds.

The fatty acid composition of the butanol extract of immature and ripe ANG was analysed. The quantitative derivatisation of fatty acids to fatty acid methyl esters (FAME) for the ultimate analysis using GLC was carried out using boron trifluoride-methanol reagent (BF$_3$-CH$_3$OH)

**Results and Discussion**

Antimicrobial activity of immature, ripe and spent ANG-butanol extracts of *Loligo duvauceli* are given in Table 1. Antibacterial activity of ANG-butanol extract from different stages of maturity was tested against three gram negative and two gram positive bacterial strains. In *L. duvauceli*, it was found that the ANG-butanol extract from ripe animals showed

<table>
<thead>
<tr>
<th>Stages of maturity</th>
<th><em>Escherichia coli</em></th>
<th><em>Aeromonas hydrophila</em></th>
<th><em>Pseudomonas aeruginosa</em></th>
<th><em>Staphylococcus aureus</em></th>
<th><em>Bacillus megaterium</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Immature</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>Ripe</td>
<td>7 mm</td>
<td>Nil</td>
<td>7 mm</td>
<td>7 mm</td>
<td>Nil</td>
</tr>
<tr>
<td>Spent</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>Control*</td>
<td>30 mm</td>
<td>40 mm</td>
<td>12 mm</td>
<td>42 mm</td>
<td>32 mm</td>
</tr>
<tr>
<td>Control**</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
</tbody>
</table>

*Chloramphenicol-Positive control

**Butanol-Negative control
antibacterial activity against *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The activity correlated well with the ripe stage of females collected during the peak period of spawning. Extracts from the immature and spent stage glands did not show any antibacterial activity.

The ANG-butanol extract of *L. pealei* could inhibit the growth of marine pathogens like *Vibrio anguillarum* and *Streptomyces griseus*. Antibacterial activity in the ANG-butanol extracts of *Sepia aculeata*, *S. pharaonis* and *Sepiella inermis* against *Escherichia coli*, *Aeromonas sp.*, *Staphylococcus aureus*, and *Bacillus megaterium* have been reported. Among the cuttlefish species, extract from *S. aculeata* showed the highest activity and among the different organic solvents used, the butanol extract gave the highest activity.

The absorption spectra of the extracts from the immature, ripe and spent accessory nidamental glands are given in Table 2. Spectrophotometric analysis of ripe ANG-butanol extract showed an absorbance maximum at 498.5 nm, followed by shoulders at 528.0, 290.5 and 315.5 nm. The absorbance spectrum of pigments extracted from the accessory nidamental glands of *L. duvauceli* corresponded with that reported for *L. pealei* and for *L. forbesi*. The peak at 498.5 nm (2.597A), seen only in the ripe stage could be attributed to the presence of carotenoid pigments which impart the orange red colour to the ripe gland. In the adult female cuttlefish, *Sepia officinalis* an orange red pigment was found concentrated in the ANG. The new carotenoid pigment was termed ‘sepiaxanthine’. The red coloration of the ANG of sexually mature squid, *L. pealei* was attributed to the bacteria resident within it. Symbiotic bacteria (*Alteromonas* strain) in *L. pealei* were orange-red pigmented.

The major peak obtained at 286 nm for the immature and spent stages indicate the presence of peptides/proteins.

Thin layer chromatography of the butanol extract from the ANG of *L. duvauceli* did not show the presence of polyphenols/flavonoids or quinones, whereas lipid components such as phospholipids, cholesterol, free fatty acids, triglycerides, fatty acid esters, and cholesteryl esters were detected (Fig. 2).

The results of the free fatty acid estimation indicated that FFA content was significantly higher (at 1% level, N=6) in ripe glands (16.0 ± 0.143 mg oleic acid/g tissue) when compared to immature ones (10.3 ± 0.114 mg oleic acid/g tissue). FFAs are known to have antimicrobial activity against various microorganisms. In cuttlefish, *S. pharaonis*, FFA content of the ANGs in different maturity stages increased with advancement in maturity. Within the ripe glands, those with higher color intensity showed higher FFA content and correspondingly higher antibacterial activity than those with lower color intensity.

The results of the fatty acid composition of the immature and ripe stages based on GLC are given in Table 3. The total fatty acid content was 3.925 mg/g tissue in ripe stage and 0.704 mg/g tissue in immature stage showing the high gross value in the ripe stage.

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**Table 2—λ max for different peaks of absorbance of pigments from ANG extracts of *Loligo duvauceli***

<table>
<thead>
<tr>
<th>Maturity stages of ANG</th>
<th>λ max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immature</td>
<td>285.0 (0.871A)</td>
</tr>
<tr>
<td>Ripe</td>
<td>290.5 (1.314A)</td>
</tr>
<tr>
<td>Spent</td>
<td>286.5 (0.862A)</td>
</tr>
</tbody>
</table>

Values within the brackets indicate the respective absorbance.
showed no or weak antibacterial activity against mutans streptococci has been reported. They found that the antibacterial activity of unsaturated fatty acids might be an alteration of cell membrane properties or generation of free radicals.

The ripe stage revealed a high proportion of unsaturated fatty acids especially docosahexaenoic acid, oleic acid, arachidonic acid and eicosapentaenoic acid (total-1.973 mg/g tissue) when compared to the immature stage (0.251 mg/g tissue).

The antibacterial activity of the extract from dried marine alga, Gloioptelis furcata against mutans streptococci has been reported. They found that the extract was a mixture of fatty acids mainly eicosapentaenoic acid (66.4%), palmitic acid (15.9%) and oleic acid (7.5%) and that they inhibited the growth of the bacteria. They also found that the unsaturated fatty acids showed more potent inhibition against insoluble glucan production than saturated fatty acids. A study on the effects of long-chain fatty acids and fatty alcohols revealed that most unsaturated fatty acids showed potent antibacterial activity while most of the saturated fatty acids showed no or weak antibacterial activity against Streptococcus mutans. The possible mechanism of antibacterial action of unsaturated fatty acids might be an alteration of cell membrane properties or generation of free radicals.

### Table 3—Fatty acid composition of ANG-butanol extracts of Loligo duvauceli

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Immature</th>
<th>Ripe</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>0.002</td>
<td>ND</td>
</tr>
<tr>
<td>C10</td>
<td>ND</td>
<td>0.004</td>
</tr>
<tr>
<td>C12</td>
<td>ND</td>
<td>0.002</td>
</tr>
<tr>
<td>C14</td>
<td>0.022</td>
<td>0.155</td>
</tr>
<tr>
<td>C14:1</td>
<td>ND</td>
<td>0.053</td>
</tr>
<tr>
<td>C15</td>
<td>0.007</td>
<td>0.024</td>
</tr>
<tr>
<td>C15:1</td>
<td>ND</td>
<td>0.019</td>
</tr>
<tr>
<td>C16</td>
<td>0.130</td>
<td>0.867</td>
</tr>
<tr>
<td>C16:1</td>
<td>0.012</td>
<td>0.097</td>
</tr>
<tr>
<td>C17</td>
<td>0.010</td>
<td>0.062</td>
</tr>
<tr>
<td>C17:1</td>
<td>0.009</td>
<td>0.013</td>
</tr>
<tr>
<td>C18</td>
<td>0.059</td>
<td>0.360</td>
</tr>
<tr>
<td>C18:1n-9</td>
<td>0.068</td>
<td>0.657</td>
</tr>
<tr>
<td>C18:2n-6</td>
<td>0.007</td>
<td>0.055</td>
</tr>
<tr>
<td>C18:3n-6</td>
<td>0.102</td>
<td>0.004</td>
</tr>
<tr>
<td>C20</td>
<td>0.013</td>
<td>0.103</td>
</tr>
<tr>
<td>C20:1</td>
<td>0.028</td>
<td>0.086</td>
</tr>
<tr>
<td>C20:2</td>
<td>ND</td>
<td>0.014</td>
</tr>
<tr>
<td>C20:3n-6</td>
<td>0.032</td>
<td>ND</td>
</tr>
<tr>
<td>C20:4</td>
<td>ND</td>
<td>0.331</td>
</tr>
<tr>
<td>C20:5n-3</td>
<td>0.056</td>
<td>0.305</td>
</tr>
<tr>
<td>C22:2</td>
<td>0.008</td>
<td>0.034</td>
</tr>
<tr>
<td>C22:6n-3</td>
<td>0.127</td>
<td>0.680</td>
</tr>
<tr>
<td>C24</td>
<td>0.012</td>
<td>ND</td>
</tr>
<tr>
<td>Total</td>
<td>0.704</td>
<td>3.925</td>
</tr>
</tbody>
</table>

(ND – Not detected)

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