CHAPTER 3

Effect of lunar periodicity on the hemagglutination activity and biochemical constituents in the extract of marine algae, S. wightii and S. cristaefolium

Abstract

On different days of the lunar cycle, samples of S. wightii and S. cristaefolium were collected and the HA activity, protein and calcium content of the different parts were studied and their inter-relationships were analyzed. In both the algae a significant positive correlation was observed between the protein content and the HA titer. It was interesting to note that the various parts of both the algae analyzed showed maximum protein and HA activity on the 21st day of the lunar cycle. However, the calcium content showed neither significant variation nor any influence on the HA titer following lunar periodicity.

Introduction

In the previous chapter, the presence of hemagglutinins in the extract of all the marine algae has been reported. Of the fifteen species of algae belonging to three different classes studied, S. wightii and S. cristaefolium showed maximum hemagglutination. Hence these two species were selected for further study on characterization and purification. The important findings observed in these two algae are that the HA activity differed from one part of the alga to the other part revealing the presence of maximum agglutinin activity in the receptacle. In addition, variation in HA titer was also observed in the samples collected on different days. This may be because the organisms of the marine littoral zone experience a much wider range of periodicity in their environment than do their terrestrial counterparts. Tidal cycles of semidiurnal, diurnal, lunar and semilunar frequencies may occur at the
same locality (Morgan, 1991). Periodic interaction of the diurnal and tidal cycles may account for a semilunar periodicity. The new moon and the full moon appear once in each lunar month of 29.5 days (Kinne, 1970). Very little is known concerning the existence of rhythmicities with periods that match either the lunar month or the semilunar spring and neap tides.

During the past years, extensive studies have been conducted on lunar and semilunar rhythms in animals. But little work has been conducted on the effect of lunar rhythms on the large and diverse groups of marine algae. As for marine algae, the green algae, Derbesia (Ziegler-Page and Kingsbury, 1968), Ulva (Smith, 1947), Enteromorpha (Christie and Evans, 1962) and the brown alga, Dictyota (Hoyt, 1927; Bunning and Muller, 1961) discharge gametes only at the very low tides (new moon). Muller (1962) and Vielhaben (1963) observed that gametes were produced in bursts that occurred every 14-15 days in Dictyota dichotoma. A cycle in reproduction continued to be expressed in which a maximum occurred every 15 days and this response could conceivably be due to semilunar periodicity. Hollenberg (1936) reported that the green alga, Derbesia forms gametangia only at especially low “spring” tides twice each lunar month. Yentsch et al. (1980) studied an annual rhythm in the growth rate of the marine dinoflagellate, Gonyaulax tamarensis. Annual rhythm in the growth of the green algae, Ankistrodesmus braunii, Chlorella pyrenoidosa (Kessler and Czygan, 1963; Kessler and Langner, 1962) has been reported.

Due to a lack of collateral information on growth and reproduction, physiological explanations to the lunar periodicity effect on marine algae cannot be developed. The only reported seasonal variation of
hemagglutination activity is that of the red alga, *Gracilaria verrucosa* (Takahashi and Katagiri, 1987). Seasonal variation in the biochemical composition of marine algae has been demonstrated for a number of species such as *Eucheuma* (Dawes et al., 1974), *Sargassum* (Prince and Daly, 1981), *Hypnea* (Durako and Dawes, 1980), *Codium* (Hanisak, 1979) and *Gracilaria* (Bird, 1984). Despite a great deal of knowledge on seasonal variation in biochemical constituents, the effect of lunar periodicity on biochemical constituents and hemagglutinating activity in marine algae is still lacking. Hence an attempt has been made to study the effect of lunar periodicity on the HA activity and biochemical constituents in the extracts of marine algae, *S. wightii* and *S. cristaefolium*.

**Material and Methods**

**Algae specimens**

Two representative species of *Sargassum*, *S. wightii* and *S. cristaefolium* were collected during a lunar cycle (at an interval of 3 days for a period of one month) from Muttom sea coast, Kanyakumari District, Tamil Nadu, South India. After collection, the associated fauna and other contaminants were removed and the algal specimens were washed thoroughly in seawater for the study. Fresh and healthy parts of algae (holdfast, stem, leaf, air bladder and receptacle) were separated carefully using forceps and thoroughly washed in seawater. Extract was prepared for different parts of algae separately and further analyzed.
Chemicals

The Folin-phenol reagent, chloranilic acid and tetrasodium EDTA were obtained from Sigma. Ethanol, sodium hydroxide, sodium carbonate, copper sulphate, sodium or potassium tartarate, isopropyl alcohol and ferric chloride were purchased from BDH.

Extract Preparation

The extract for different parts of both species of algae were prepared as stated in chapter 2.

Erythrocyte collection

Pig erythrocytes were used for the hemagglutination assay. Blood for this purpose was obtained from slaughterhouse. Erythrocytes were collected directly in modified Alsevier’s medium (containing Sodium citrate: 30 mM, pH 6.1; NaCl: 77 mM, glucose: 114 mM; neomycin sulphate: 100 µg/ml, Chloramphenicol: 330 µg/ml). They were suspended and washed three times with ten volumes of Tris-buffered saline and resuspended in TBS as 1.5 % suspension.

Hemagglutination assay

HA assay for different parts of both species of algae, S. wightii and S. cristaefolium were carried out as stated in Chapter 2.

Estimation of protein

The protein concentration was estimated by Folin-Ciocalteau method (Lowry et al., 1951). The principle of this method involves two steps.
(i) The carbamyl groups of protein molecules react with copper and potassium of the biuret reagent to give a blue coloured copper potassium-biuret complex.

(ii) This complex together with tyrosine and phenolic compounds present in the protein reduce the phospho molybdate of the folin reagent to intensify the colour of the solution.

0.05 ml of the extract was poured into 1 ml of deproteinizing agent (80 % ethanol) using a fine graduated micropipette. This mixture was centrifuged at 3000 rpm for 5 min and the precipitate was dissolved in 1 ml of 1 N NaOH. To this 5 ml of reagent mixture (50 ml of reagent A: 2 g of Na₂CO₃ in 100 ml of 0.1 N NaOH and 1 ml of reagent B: 500 mg of CuSO₄ in 1 % sodium or potassium tartarate) was added and mixed well for 10 min. Then 0.5 ml of Folin-phenol reagent (freshly prepared 1:1 ratio) was added mixed rapidly and incubated for 30 min absorbency was measured at 500 nm.

**Estimation of Calcium**

The calcium was estimated by Webster’s spectrophotometric method (Webster, 1962). Chloranilic acid (2,5 dichloro 3, 6 dihydroxy P-quinone compound L111) precipitates the calcium present in the extract forming a calcium chloranilate complex. This precipitate was dissolved in tetrasodium EDTA that liberates the chloranilic acid. The liberated chloranilic acid combined with ferric chloride to form a coloured complex that was measured at 490 nm in a spectrophotometer. The amount of liberated chloranilic acid is directly proportional to the amount of calcium precipitated.
Extract was used directly for determination of total calcium. Chloranilic acid (0.1 ml) was added to 0.1 ml of extract or 2 ml of ethanolic supernatant or 0.1 ml of calcium standard solution. It was mixed and allowed to stand for at least one hour at room temperature. The suspension was centrifuged at 900 x g for 10 min and the supernatant was decanted. The tubes were drained by keeping it inverted on a filter paper for 5 min. To this 5 ml of 50 % isopropyl alcohol was added and centrifuged at 700 x g for 5 min and the supernatant was decanted. Two drops of 5 % tetra sodium EDTA were added and the precipitate was broken by striking the bottom of the tube forcibly against a rubber stopper. Finally 5 ml of 0.6 % ferric chloride solution was added and mixed well by agitation or inversion and kept it for 5 min. The absorbency was determined at 490 nm in a spectrophotometer.

Results

Presence of agglutinins in different part of algae

Agglutinins were found in the extracts of all parts (holdfast, stem, leaf, air bladder and receptacles) of the algae, *S. wightii* and *S. cristaefolium*. On different days of the lunar cycle the HA titer ranged from 32-128 in holdfast, 16-128 in stem, 16-256 in leaf, 32-128 in air bladder and 128-4096 in receptacle of *S. wightii* (Table 3.1 & fig 3.1) and 32-256 in holdfast, 16-128 in stem, 32-512 in leaf, 32-256 in air bladder and 128-8192 in receptacle of *S. cristaefolium* (Table 3.2 & fig 3.2). The holdfast, stem, leaf, air bladder and receptacles of both *S. wightii* and *S. cristaefolium* showed maximum HA titer on the 21st day of the lunar cycle (Table 3.7 and Figure 3.7). Among the different parts assayed the receptacles showed highest hemagglutination activity in both the
species of algae (Table 3.1 & 3.2). The receptacles of *S. wightii* and *S. cristaefolium* showed a maximum HA titer of 1:4096 and 1:8192 respectively.

**Effect of lunar periodicity on biochemical constituents**

The present study indicates that the protein content of both algae, *S. wightii* and *S. cristaefolium* varies within a period of 30 days. The protein content of *S. wightii* falls between 8.95 and 24.76 mg protein/gm tissue. Maximum protein content was observed during the 21-24 days of the lunar cycle and relatively less protein content was observed during the period of 0-6 days (new moon) of the lunar cycle (Table 3.3). The protein content of *S. cristaefolium* varies from 6.19 to 24.73 mg protein/gm tissue during the lunar cycle. Maximum protein content was observed in all the parts studied of the alga, *S. cristaefolium* on the 21st day and the least protein content was recorded on the 9th day of the lunar cycle (Table 3.4). The studies indicate that the quantities of protein in different parts of both the species of algae undergo a periodic variation in accordance to the lunar periodicity.

The analysis of different parts of both species of algae showed the presence of calcium. Maximum calcium content was recorded for all the parts in both the species of algae on the full moon day (Fig. 3.5 & 3.6). High calcium content was observed in the stem and leaf of *S. wightii* and in stem, leaf and receptacle of *S. cristaefolium*. The calcium content of *S. wightii* receptacles ranged from 3 - 11 mg calcium/gm tissue and the *S. cristaefolium* ranged from 3 - 13 mg calcium/gm tissue during the lunar cycle. The holdfast of both the species of algae showed less calcium content (Table 3.5 & 3.6). The results indicate that the lunar
cycle does not seem to have a significant influence on the calcium content of the various parts of algae.

**Discussion**

**Possible significance of HA activity of receptacle and other tissues**

The effect of lunar periodicity on HA, protein and calcium content of the different parts of the marine algae, *S. wightii* and *S. cristaefolium* were analyzed through one full lunar cycle. The various parts of both the species of algae showed maximum HA activity on the 21st day of the lunar cycle. The result of this investigation showed significant variation in protein content in the various parts the algae. Similar results were reported for the marine algae, *Ulva rigida, Dictyota maxima, Padina tetrastromatica* (Krishnamurthy, 1967). It can be conveniently concluded that the HA activity and protein content of the algae were under the direct influence of lunar periodicity. The calcium content in the various parts of the algae, *S. wightii* and *S. cristaefolium* fluctuated considerably that has no reasonable correlation with the lunar cycle. It can be concluded that calcium utilization in algae was independent of the lunar effect.

The moon on the mid-day that lies between the new moon and full moon day emits this rhythmic fluctuation because of the light intensity. In some localities it has been shown experimentally that light intensities equivalent to moonlight are sufficient to entrain such rhythms (Naylor, 1985). Circa semilunar and circalunar periodicity have been demonstrated in a number of species (Naylor, 1985). Majority of these works on lunar periodicity lies on reproduction and spawning (Hauenschild, 1960; Hauenschild et al., 1968; Walker, 1952), maturation
of gonad (Korringa, 1957), animal movement (Horning and Trillmich, 1999; Wikelski and Hau, 1995; Savage and Hodgson, 1934; Boetius, 1967) and eclosion of insects (Neumann, 1989). According to (Neumann, 1976), the semilunar periodicity differ in different populations depending upon local tide conditions. Similarly the protein and agglutinin content of the algae, *S. wightii* and *S. cristaefolium* are highly influenced by the tidal periodicity.

Although most of the activities of the organisms are influenced by the lunar periodicity, it is surprising to note that the calcium content of the various parts of the algae is not influenced by the lunar or semilunar periodicity. It might be due to the abundance of calcium available in the marine environment, which could be utilized by the marine algae according to its need.

**Future directions**

For further characterization of the marine algal agglutinin, observations are restricted to the receptacles of *S. cristaefolium* collected on the 21st day of the lunar cycle, because the hemagglutinating efficiency of the extract of the receptacles of *S. cristaefolium* is greater to that of different parts tested and maximum HA was observed on the 21st day of the lunar cycle.

**Salient findings**

The important findings emerge from this study are

1. Various parts of both the species of algae, *S. wightii* and *S. cristaefolium* showed the effect of lunar periodicity on the HA titers and protein content.
2. Receptacles of both the species of algae showed maximum HA activity.

3. The HA and protein content of different parts of both species of algae assayed showed maximum activity on the 21st day of the lunar cycle.

4. The calcium content of the various parts does not seem to have a significant influence on the HA activity.
Table 3.1 Effect of lunar periodicity on the HA titer of different parts of the alga, *S. wightii*.

Samples were collected on different days of lunar cycle and the different parts of the alga, *S. wightii* were analyzed for HA activity against pig erythrocytes. HA assay was determined as stated in Chapter 2.

<table>
<thead>
<tr>
<th>Lunar cycle in days</th>
<th>HA titer (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Holdfast</td>
</tr>
<tr>
<td>0</td>
<td>32</td>
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<tr>
<td>3</td>
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<td>6</td>
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<td>9</td>
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</tr>
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<td>27</td>
<td>64</td>
</tr>
<tr>
<td>FM 30</td>
<td>32</td>
</tr>
</tbody>
</table>

n = number of algal samples

NM – New moon.          FM - Full moon
Table 3.2 Effect of lunar periodicity on HA titer of different parts of the alga, *S. cristaefolium*.

Samples were collected on different days of lunar period and the different parts of the alga, *S. cristaefolium* were analyzed for HA activity against pig erythrocytes. HA assay was determined as stated in chapter 2.

<table>
<thead>
<tr>
<th>Lunar cycle in days</th>
<th>HA titer (n = 10)</th>
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<tr>
<td></td>
<td>Holdfast</td>
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<tr>
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<td>32</td>
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</tr>
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<td>27</td>
<td>64</td>
</tr>
<tr>
<td>FM 30</td>
<td>32</td>
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</tbody>
</table>

n = number of algal samples

NM - New moon        FM - Full moon
Table 3.3 Effect of lunar periodicity on the protein content of different parts of the alga, *S. wightii*.

Protein content in different parts of the alga was estimated by Folin-Ciocalteau method (Lowry et al., 1951).

<table>
<thead>
<tr>
<th>Lunar cycle in days</th>
<th>Protein content (mg protein/gm tissue) (n = 10)</th>
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<td></td>
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<tr>
<td>0</td>
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<td>15.79</td>
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</tr>
<tr>
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<td>19.29</td>
</tr>
<tr>
<td>FM 30</td>
<td>17.60</td>
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</table>

*n* = number of algal samples

NM – New moon.        FM - Full moon
Table 3.4 Effect of lunar periodicity on the protein content of different parts of the alga, *S. cristaefolium*.

Protein content in different parts of the alga was estimated by Folin-Ciocalteau method (Lowry et al., 1951).

<table>
<thead>
<tr>
<th>Lunar cycle in days</th>
<th>Protein content (mg protein/gm tissue) (n = 10)</th>
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<td>13.16</td>
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n = number of algal samples

NM - New moon. FM - Full moon
Calcium content in different parts of the alga was estimated by Webster's Spectrophotometric method (Webster, 1962).

<table>
<thead>
<tr>
<th>Lunar cycle in days</th>
<th>Calcium content (mg calcium /gm tissue) (n = 10)</th>
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<tbody>
<tr>
<td></td>
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</tr>
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<tr>
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<tr>
<td>FM 30</td>
<td>4</td>
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</tbody>
</table>

n = number of algal samples

NM - New moon.  
FM - Full moon
Table 3.6 Effect of lunar periodicity on the calcium content of different parts of the alga, S. cristaefolium.

Calcium content in different parts of the alga was estimated by Webster’s Spectrophotometric method (Webster, 1962).

<table>
<thead>
<tr>
<th>Lunar cycle in days</th>
<th>Calcium content (mg calcium /gm tissue) (n = 10)</th>
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<td></td>
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n = number of algal samples

NM - New moon.        FM - Full moon
Table 3.7 Peak of agglutinin activity in various parts of the algae, *S. wightii* and *S. cristaefolium* on the 21\textsuperscript{st} day of the lunar cycle.

Various parts of the algae, *S. wightii* and *S. cristaefolium* were collected on the 21\textsuperscript{st} day of the lunar cycle when the hemagglutination activity was at its peak. The HA assay was determined as stated in chapter 2.

<table>
<thead>
<tr>
<th>Parts of algae</th>
<th>HA titer (n = 10)</th>
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<tbody>
<tr>
<td></td>
<td><em>S. wightii</em></td>
<td><em>S. cristaefolium</em></td>
</tr>
<tr>
<td>Hold fast</td>
<td>128</td>
<td>256</td>
</tr>
<tr>
<td>Stem</td>
<td>128</td>
<td>128</td>
</tr>
<tr>
<td>Leaf</td>
<td>256</td>
<td>512</td>
</tr>
<tr>
<td>Air bladder</td>
<td>128</td>
<td>256</td>
</tr>
<tr>
<td>Receptacle</td>
<td>4096</td>
<td>8192</td>
</tr>
</tbody>
</table>

n = number of algal samples.
Table 3.8 Amount of protein in various parts of the algae *S. wightii* and *S. cristaefolium* on the 21\textsuperscript{st} day of the lunar cycle.

Various parts of the algae, *S. wightii* and *S. cristaefolium* were collected on the 21\textsuperscript{st} day of the lunar cycle where the HA activity was high, to test the protein content. Protein content in different parts of algae were estimated by Folin-Ciocalteau method (Lowry et al., 1951).

<table>
<thead>
<tr>
<th>Parts of algae</th>
<th>Protein content (mg protein /gm tissue) (n = 10)</th>
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<tbody>
<tr>
<td></td>
<td><em>S. wightii</em></td>
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<tr>
<td>Hold fast</td>
<td>20.48</td>
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<tr>
<td>Stem</td>
<td>21.05</td>
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<tr>
<td>Leaf</td>
<td>23.00</td>
</tr>
<tr>
<td>Air bladder</td>
<td>15.79</td>
</tr>
<tr>
<td>Receptacle</td>
<td>24.38</td>
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</table>

\(n=\) number of algal samples.
Fig 3.1 Effect of lunar periodicity on the HA titer of different parts of the alga, *Sargassum wightii*
Fig 3.2 Effect of lunar periodicity on the HA titer of different parts of the alga, *Sargassum cristaefolium*
Fig 3.4 Effect of lunar periodicity on the protein content of different parts of the alga, *S. cristaefolium*
Fig 3.5 Effect of lunar periodicity on the calcium content of different parts of the alga, *S. wightii*
Fig 3.6 Effect of lunar periodicity on the calcium content of different parts of the alga, *S. cristaefolium*
Fig 3.7 Peak of agglutinin activity in various parts of the algae, S. wightii and S. cristaefolium on the 21st day of the lunar cycle.
Fig 3.8 Amount of protein in various parts of the algae, *S. wightii* and *S. cristaeefolium* on the 21st day of the lunar cycle.

<table>
<thead>
<tr>
<th>Parts of algae</th>
<th>Protein content (mg protein/gm tissue)</th>
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<tbody>
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<td>Hold fast</td>
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<tr>
<td>Leaf</td>
<td>20</td>
</tr>
<tr>
<td>Air bladder</td>
<td>15</td>
</tr>
<tr>
<td>Receptacle</td>
<td>25</td>
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</tbody>
</table>

![Graph showing protein content in various parts of the algae](image-url)