Dietary Effect of *Porphyra* Spheroplasts for Short-neck Clams: A Preliminary Report

Alok Kalla¹, Takao Yoshimatsu²,*, Mohammad Nakib Dad Khan¹, Junya Higano², Toshiyoshi Araki¹ and Shuichi Sakamoto³

Abstract: We investigated preliminarily the availability of *Porphyra* spheroplasts (PS) as a live food substitute for culturing bivalve, *Ruditapes philippinarum*. Hatchery obtained clam spats (mean shell length ca. 5 mm) were randomly distributed in small stainless-steel mesh cages at 20 pieces per cage with six replicates of each dietary treatment. Two diets (PS and condensed natural diatom *Chaetoceros gracilis*) were fed to the clams for 42 days, four times a day (08:00, 12:00, 16:00 and 20:00) at *ad libitum* in a flow through system. In addition, adult clams (mean shell length ca. 29.8 mm) were reared in the same condition in plastic cages at 20 pieces per cage with three replicates. The dietary performance was evaluated in terms of survival, growth of shell size and body composition for clams. The data on length increase indicated that the clam spats fed on *C. gracilis* had higher increment in size than those fed on PS. Nevertheless, over-all mortality was low (survival: 97.5 – 99.2%) and independent of the experimental treatments. In addition, no significant difference was observed in proximate carcass composition after feeding trial for adult clams. According to the results, PS was proved to be a good candidate food substitute for culturing bivalve spats.

Key words: Short-neck clam; *Porphyra* spheroplasts; Feed; Growth; Body composition

The short-neck clam *Ruditapes philippinarum* is among the important seafood and an item of profitable capture fishing in Japan. The short-neck clam culture is a key and rapidly rising area of Japanese aquatic production. The greater part of the shellfish production is from natural populations while increasingly culture seeds are imminent or have exceeded utmost sustainable yields. Stock improvement through the capture and imparting of natural seed in both extensive and intensive forms of culture is frequent practice. The regularity of natural recruitment can never be certain. An elucidation to meet the seed requirements of the short-neck clam is hatchery culture. The production of seed through hatchery propagation accounts at the present for only a small percentage may only be attributed to unavailability of artificial-supplementary feed. There is an intense interest in the aquaculture of short-neck clam all over the Japan due to their high significance in local markets and because most stocks are exploited at or above sustainable levels (Toba 2005). Although short-neck clam larvae and spat have been reared successfully on microalgal diets, these are expensive to produce and do not always coordinate with producer’s requirements (Toba 2005). However, little attention paid by researchers to investigate substitute to microalgal feeds. Expensive microalgal concentrates, used throughout the world (Whyte 1987), have overcome some constrain associated with self-life of microalgal concentrate and storage of algal feeds. Hitherto, studies on *R. philippinarum*, in the laboratory, have not included any supplementary feed by using *Porphyra* except those carried out (Yoshimatsu
et al. 2007). Furthermore, few studies have dealt with possible effects of supplementary feeding while using dietary diatoms on the composition of adult and spat individual of this species (Caers et al. 1998).

Druehl (2000) stated that “Nori (Porphyra) is probably one of the healthiest foods on our planet”. However, cells of seaweeds are covered with a cell wall which makes it hard to digest. We have been, therefore, tried to develop the feed for rearing shellfish by using spheroplasts (PS) from Porphyra product, which were prepared by digesting the cell wall with three kinds of cell wall-degrading enzymes, and to determine the effect of PS for growth and production of culture short-neck clam. Recently the efficient biochemical technology for mass-production of Porphyra spheroplasts and protoplasts using polysaccharases isolated from bacteria was developed (Araki et al. 1998, 1999). Spheroplasts are cells that have had their cell wall partially removed using enzymatic means. As there is no report on spheroplasts in short-neck clam, we investigated preliminarily the availability of Porphyra spheroplasts as a live food substitute for culturing short-neck clams.

**Materials and Methods**

*Isolation of spheroplasts*

The dried thalli of Porphyra were supplied from Saga Prefectural Ariake Fisheries Research and Development Center. The dried thalli of Porphyra (1 kg) were immersed in the cell wall-degrading enzyme solution which contained three kinds of enzymes (60 g Sumizyme ACH, 200 units of agarase, 100 units of $\beta$-1, 3-xylanase) into 30 l of water and agitated at 20°C for 20 h. The products collected by centrifugation (3,000 $\times$ g, 10 min) were lyophilized and named as spheroplasts (PS). Sumizyme ACH having $\beta$-mannanase activity was purchased from Shin Nihon Chemical Co. Ltd (Japan). Agarase and $\beta$-1, 3-xylanase were prepared from culture fluids of *Vibrio sp.* PO-303 and XY-214, respectively, by the method described previously (Araki et al. 1998, 1999). One unit is defined as amount of each enzyme that produces reducing sugar corresponding to the constituent monosaccharide of each substrate polysaccharide (agar and $\beta$-1, 3-xylan). After getting PS, it was subjected to smash into powder form manually by mortar.

**Experimental diets and design**

The spats and adults of short-neck clams were obtained from hatchery and market in Mie Prefecture, respectively. The clam spats (mean shell length ca. 5 mm) were randomly distributed in circular stainless-steel mesh cages (size: 8 cm in diameter) at 20 pieces per cage with six replicates and the adult clams (mean shell length ca.29.8 mm) were maintained in plastic cages (size: 22 $\times$ 15 cm) at 20 pieces per cage with three replicates. Each aquarium was aerated (400 – 600 ml/min) and supplied with a flow-through thermo-controlled seawater (32 – 34 psu, 20°C, 8 l/min) under the photo-condition of 12L : 12D. Two diets, PS and condensed natural microalga, *Chaetoceros gracilis*, were fed to the spat and adult clams four times a day (08:00, 12:00, 16:00 and 20:00) at ad libitum in a flow through system for 42 days. Before feeding, PS was mixed with seawater using mixer and offered to clam in liquid form by pipette.

All the experimental short-neck clams were measured individually at bi-weekly intervals. At the end of the feeding trials, we checked shell flesh weight, length, breadth, height and survival. Proximate analyses for flesh of adult short-neck clam were carried out from pooled samples of each group obtained after the termination of the experiment. The condition index used for evaluating rearing results was calculated as follows;

$$\text{Condition Index} = \frac{\text{FW} \times 100}{(\text{SL} \times \text{SB} \times \text{SH})}$$

Where, FW: flesh wet weight (g), SL: shell length (cm), SB: shell breadth (cm), SH: shell height (cm).

**Proximate composition**

Determinations of moisture, crude protein, crude fat, and crude ash of the PS and short-neck clam were made from triplicate samples by 10 h drying at 110°C, semi-micro Kjeldahl method (N $\times$ 6.25), ethyl ether extraction, and 5 h combustion at 600°C, respectively. Total lipids were extracted using a modified procedure (Folch et al. 1957). Lipid was saphonified
Porphyra Spheroplasts as Short-neck Clam Feed

using 2N KOH in methanol, and fatty acids were converted to methyl esters with 5% H₂SO₄ in methanol. These fatty acid methyl esters were diluted in hexane and analyzed on a Shimadzu fused silica capillary column (CPB20-M25-025) in a Shimadzu 2010 GC with a FID detector (Kyoto, Japan). The temperature program was 150°C for 3 min; followed by an increase at a rate of 3°C/min to a final temperature of 220°C for 15 min. Peaks were identified by comparison to GLC standard mixtures (SUPELCO, Bellefonte, PA). Statistical analyses were performed using a Student’s t-test and 5% level was considered statistically significant.

Results

Nutritional profile of PS

Proximate compositions of unprocessed dried Porphyra and PS (Table 1) were revealed that after enzymatic process of Porphyra its protein and lipid contents (36.7% and 2.9%, respectively) increased from that before treatment (29.1% and 0.1%, respectively) due to the decomposition of cell wall polysaccharides. As a result, the crude ash content decreased from 10.9% to 3.5%. Fatty acid composition of PS was rich in two unsaturated acids: eicosapentaenoic acid (EPA, C20:5n-3) and arachidonic acid (C20:4n-6), and a saturated acid: palmitic acid (C16:0) (Table 2).

Growth performance of short-neck clam spats

The comparison in growths of short-neck clam spats fed on PS and Chaetoceros gracilis were shown in Fig. 1. The shell length, breadth and height of the

Table 1. Proximate composition of dried Porphyra and Porphyra spheroplasts

<table>
<thead>
<tr>
<th>Parameters (%)</th>
<th>Dried Porphyra</th>
<th>Porphyra spheroplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>8.4 ± 0.5</td>
<td>5.9 ± 0.1</td>
</tr>
<tr>
<td>Crude Protein</td>
<td>29.1 ± 0.2</td>
<td>36.7 ± 0.7</td>
</tr>
<tr>
<td>Crude Lipid</td>
<td>0.1 ± 0.0</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>Crude Ash</td>
<td>10.9 ± 0.1</td>
<td>3.5 ± 0.1</td>
</tr>
</tbody>
</table>

Mean ± SE (n=3).

Table 2. Fatty acid composition (% in total fatty acid) of Porphyra spheroplasts

<table>
<thead>
<tr>
<th>Fatty acids (area %)</th>
<th>Porphyra spheroplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>0.4</td>
</tr>
<tr>
<td>C16:0</td>
<td>24.9</td>
</tr>
<tr>
<td>C16:1n-7</td>
<td>1.1</td>
</tr>
<tr>
<td>C18:0</td>
<td>0.9</td>
</tr>
<tr>
<td>C18:1n-9</td>
<td>2.8</td>
</tr>
<tr>
<td>C18:1n-7</td>
<td>0.6</td>
</tr>
<tr>
<td>C18:2n-6</td>
<td>1.2</td>
</tr>
<tr>
<td>C18:3n-3</td>
<td>0.2</td>
</tr>
<tr>
<td>C18:4n-3</td>
<td>0.6</td>
</tr>
<tr>
<td>C20:1</td>
<td>4.0</td>
</tr>
<tr>
<td>C20:4n-6</td>
<td>10.1</td>
</tr>
<tr>
<td>C20:5n-3</td>
<td>34.9</td>
</tr>
<tr>
<td>C22:1</td>
<td>2.4</td>
</tr>
<tr>
<td>C22:5n-3</td>
<td>0.4</td>
</tr>
<tr>
<td>C22:6n-3</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Fatty acid contents were expressed as a mean percentage (n=2) of the total area identified in the chromatograms, unidentified peaks were not considered in the calculation.

Fig. 1. Biweekly (a) length, (b) breadth, and (c) height increments of short-neck clam spats. Values are mean ± SE (n=6).
short-neck clam spat fed on PS diets increased 1.25, 1.27, and 1.37 times after 42 days of rearing period. However, the growth of PS diet group was not good compared with those of C. gracilis diet group of which the increment of shell length, breadth and height was 1.51, 1.49, and 1.63 times. The both diet group exhibited high survival rate (97.5 – 99.2%).

**Growth performance and proximate composition of adult short-neck clams**

The adult short-neck clams were reared for 42 days by PS and C. gracilis, and condition index of each diet group was compared. Condition index of PS diet group was lower than that of C. gracilis group (Fig. 2). The survival rates of PS and C. garacilis diet groups were 63.3% and 86.7%, respectively.

The proximate compositions of body carcase of adult short-neck clams among initial and final PS and C. gracilis fed groups are shown in Table 3. No significant (P > 0.05) difference was observed in final carcase proximate composition of adult short-neck clams, although slight increase was noted in crude protein contents between initial and final groups. Fatty acids composition of the body carcase of clams fed on two diets were summarized in Table 4. Overall, there observed quite large difference in fatty acid compositions between two dietary groups. Namely, PS fed clams contained very low fatty acids like C14:0, C16:1n-7, C16:3n-3, C18:3n-6, C18:4n-3 and C22:0, and conversely high in C18:0, C18:1, C20:2n-6, C20:4n-6, C22:1,C22:4n-6, C22:5n-3 and DHA (C22:6n-3). Lipid contents of these two dietary groups were completely identical (0.9 ± 0.1%, Table 1).

### Table 3. Proximate composition of adult short-neck clams fed on *Porphyra* spheroplasts and *C. gracilis*

<table>
<thead>
<tr>
<th>Proximate composition (%)</th>
<th>Initial values</th>
<th><em>Porphyra</em> spheroplasts</th>
<th><em>C. gracilis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>81.5 ± 0.9</td>
<td>81.4 ± 0.3</td>
<td>80.2 ± 0.5</td>
</tr>
<tr>
<td>Crude Protein</td>
<td>6.8 ± 0.2</td>
<td>7.1 ± 0.1</td>
<td>7.2 ± 0.2</td>
</tr>
<tr>
<td>Crude Lipid</td>
<td>0.8 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Crude Ash</td>
<td>2.1 ± 0.1</td>
<td>2.1 ± 0.1</td>
<td>1.9 ± 0.1</td>
</tr>
</tbody>
</table>

Mean ± SE (n=3).

### Table 4. Fatty acid composition (% in total fatty acid) of short-neck clam fed on *Porphyra* spheroplasts and *C. gracilis*

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th><em>C. gracilis</em></th>
<th><em>Porphyra</em> spheroplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>4.83 ± 0.33</td>
<td>0.27 ± 0.01**</td>
</tr>
<tr>
<td>C15:0</td>
<td>0.35 ± 0.00</td>
<td>0.32 ± 0.01*</td>
</tr>
<tr>
<td>C16:0</td>
<td>10.74 ± 0.21</td>
<td>7.89 ± 0.64*</td>
</tr>
<tr>
<td>C16:1n-7</td>
<td>13.50 ± 0.70</td>
<td>0.98 ± 0.75**</td>
</tr>
<tr>
<td>C16:3n-6</td>
<td>0.38 ± 0.09</td>
<td>1.39 ± 0.27*</td>
</tr>
<tr>
<td>C16:3n-3</td>
<td>3.65 ± 0.38</td>
<td>0.45 ± 0.18**</td>
</tr>
<tr>
<td>C18:0</td>
<td>6.05 ± 0.27</td>
<td>7.96 ± 0.28**</td>
</tr>
<tr>
<td>C18:1</td>
<td>1.65 ± 0.30</td>
<td>4.09 ± 0.02*</td>
</tr>
<tr>
<td>C18:2n-6</td>
<td>0.25 ± 0.00</td>
<td>0.35 ± 0.02*</td>
</tr>
<tr>
<td>C18:3n-6</td>
<td>1.73 ± 0.09</td>
<td>0.24 ± 0.01**</td>
</tr>
<tr>
<td>C19:0</td>
<td>0.07 ± 0.04</td>
<td>0.08 ± 0.00NS</td>
</tr>
<tr>
<td>C18:3n-3</td>
<td>0.07 ± 0.00</td>
<td>0.04 ± 0.01*</td>
</tr>
<tr>
<td>C18:4n-3</td>
<td>0.33 ± 0.01</td>
<td>0.15 ± 0.05**</td>
</tr>
<tr>
<td>C20:0</td>
<td>0.12 ± 0.00</td>
<td>0.33 ± 0.25NS</td>
</tr>
<tr>
<td>C20:1</td>
<td>2.93 ± 0.36</td>
<td>5.83 ± 0.58*</td>
</tr>
<tr>
<td>C20:2n-6</td>
<td>0.78 ± 0.11</td>
<td>3.27 ± 0.47**</td>
</tr>
<tr>
<td>C20:4n-6</td>
<td>2.86 ± 0.24</td>
<td>5.31 ± 0.39**</td>
</tr>
<tr>
<td>C20:4n-3</td>
<td>0.12 ± 0.00</td>
<td>0.20 ± 0.04*</td>
</tr>
<tr>
<td>C20:5n-3</td>
<td>12.10 ± 0.57</td>
<td>6.21 ± 1.61*</td>
</tr>
<tr>
<td>C22:0</td>
<td>0.64 ± 0.01</td>
<td>0.00 ± 0.00**</td>
</tr>
<tr>
<td>C22:1</td>
<td>0.06 ± 0.01</td>
<td>0.13 ± 0.00**</td>
</tr>
<tr>
<td>C22:4n-6</td>
<td>0.98 ± 0.14</td>
<td>2.86 ± 0.37**</td>
</tr>
<tr>
<td>C22:5n-3</td>
<td>1.37 ± 0.13</td>
<td>2.90 ± 0.37**</td>
</tr>
<tr>
<td>C22:6n-3</td>
<td>5.43 ± 0.33</td>
<td>9.45 ± 0.72**</td>
</tr>
</tbody>
</table>

**Mean ± SE (n=3).**

Fatty acid contents were expressed as mean ± SE (n=3) of the total area identified in the chromatograms, unidentified peaks were not considered in the calculation. Student’s *t*-test, *P* < 0.05, **P** < 0.01, NS not significantly different.
Porphyra Spheroplasts as Short-neck Clam Feed

Discussion

No significant differences ($P > 0.05$) in carcass proximate composition of adult short-neck clam between the dietary treatments but less growth in the clam fed PS may attribute to its probable deficiency of some nutrients. Furthermore, certain minor components such as vitamins and minerals may play an important role (Caers et al. 1998). In this respect, supplementation of PS with other nutrients could be a useful substitute for further artificial diet development studies and can contribute to a better understanding of the specific nutrient requirements.

The present Study further revealed that PS fed short-neck clam resulted in higher DHA and lower EPA than *C. gracilis* fed short-neck clam. Fatty acid composition of the diet, influence the fatty acid composition of short-neck clam (Langdon and Waldock 1981; Enright et al. 1986; Caers et al. 1998). However, the authentic efficiency of PS lipids and amino acids, which ingested and assimilated by the short-neck clam spat, is not known due to lack of $^{14}$C-labelled (Chu and Greaves 1991; Caers et al. 1998) diet method. These results have demonstrated the suitability of PS as an alternate food source for short-neck clam, when survival and body composition are taken into consideration.

Preliminary rearing trials conclude that PS can be used as a diet for short-neck clam spats as well as for growing short-neck adult clam but need to be standardized the level of feeding frequency, techniques and additives of essential nutrients in diet. Moreover, refinements of nutritionally balanced, economical and environmental friendly *Porphyra* spheroplasts diets production technology are required before application.

Acknowledgments

This study was supported by a grant program of the Agriculture, Forestry and Fisheries Research Council in Japan (Research projects for utilizing advanced technologies in agriculture, forestry, and fisheries. No.1681, 2004–2006). Author expresses the gratitude to Drs. T. Yamamoto, M. Tokuda, T. Sugita and H. Furuita of the National Research Institute of Aquaculture, for their technical advices and help.

References


アサリに対するアマノリスフェロプラストの飼料効果：予報

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日向野純也・荒木利芳・酒本秀一

アマノリ Porphyra yezoensis を原料として酵素処理によりスフェロプラストを調整し、その粉末乾燥品の飼料価値をアサリ Ruditapes philippinarum を用いて検討した。ステンレス製の小型飼育箱に20個体ずつのアサリを収容し、スフェロプラスト飼料と濃縮珪藻（Chaetoceros gracilis）を1日に4回給餌して流水下で飼育を行った。6週間の飼育試験の結果、成長は珪藻給餌区に劣るもの高い生残率が維持され（97.5－99.2%）、また体成分においても珪藻給餌区との間に差は認められず、アマノリから調整したスフェロプラスト飼料がアサリに有効であることが示された。