A New Bioassay Method for Phagostimulants for a Young Abalone, *Haliotis discus* Reeve†

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Feeding preferences or chemoreception in the feeding behavior of gastropods have been studied biologically.1 Limited numbers of chemical approaches have been made to clarify that some amino acids, propionic acid, trimethyl amine, etc. played important roles in the feeding behaviors of particular species of gastropods.

In the feeding behavior of an abalone, "Kuroawabi," *Haliotis discus* Reeve, a herbivorous gastropod, the importance of olfaction was suggested.5 Kikuchi et al. observed that an abalone, "Ezoawabi," *Haliotis discus hannai* Ino, fed on most algae, but showed their feeding preference on some kinds of seaweeds.6 Their feeding preference on brown algae were shown by examination of the stomach contents of the abalone.6,7 Some brown algae such as "Wakame," *Undaria pinnatifida* (Harvey Spingar), and "Onikonbu," *Laminaria* sp., were reported to be the most effective as their foods.6,8 On the other hand Shepherd reported that an Australian *Haliotis* sp. fed mainly on red algae.9

Harada et al. studied the attractive effect of seaweeds on the behavioral responses of a young abalone, *H. discus*, applying a logistic curve.5 Though some species of green and red algae were also found to be effective in attracting the abalone, brown ones generally tend to attract the abalone. However, there has been no paper dealing with chemical studies on the feeding behaviors of abalones.

These situation prompted us to begin chemical studies on the feeding behavior of a herbivorous gastropod, *Haliotis* sp., whose seed production has recently been carried out on a large scale at most of the Fish Farming Centers in Japan.

First of all we kept young abalones, *H. discus*, in an aquarium and observed their feeding behaviors. Careful observations allowed us to develop a new bioassay method for the feeding-stimulants for the abalones. The methanol extracts of several seaweeds were subjected to the bioassay. Among seven species of seaweeds the brown algae, *U. pinnatifida* and *Padina arborescens*, were judged to be the most active (Table I). Application of this assay procedure to the methanol extracts of *U. pinnatifida* have led us to isolate a phagostimulant for the young abalone.10 We would like to present here the details of the new, simple and reliable bioassay procedure for the feeding-stimulants for the abalones.

† Chemical Studies on Phagostimulants for Marine Gastropods. Part II.
MATERIALS AND METHODS

Test animals. About one-year-old young abalones, *H. discus*, were kindly supplied from the Shizuoka, Aichi, and Kanagawa Prefecture Fish Farming Centers. They had been kept in the experimental aquarium (Fig. 1) being fed on “Arame,” *Eisenia bicyclis* (Kjellman) Setchell, until they grew up to the desired size (20 ~ 30 mm in shell length; 0.8~3 g in weight), and were starved for at least a day before the test.

An experimental aquarium. Figure 1 shows a diagrammatic view of an experimental aquarium (a commercially available plastic planter colored dark blue, Fig. 2) with a drain. A shelter made of black polyvinyl chloride in the shape of roof (6 x 12 cm) was placed for the abalones to rest under during the day. Filtered sea-water was introduced into the aquarium through the inlet and air was introduced, too. Ten of the young abalones described above were kept in each aquarium. A piece (ca. 8 cm in length) of dry *E. bicyclis* was fed once every few days as a maintenance food. The aquarium was covered with a blue plastic board.

Preparation of the sample to be tested.

1. For the filter paper method. Methanol extracts (equivalent to 0.2 g of dried material) of *E. bicyclis* were absorbed on pieces of filter paper (5.5 cm in diameter, No. 2, Toyo Roshi Co., Ltd.). The reference paper was made by putting the same volume of methanol on the paper and drying it.

2. For the polysaccharide method. Agar powder (0.4 g) was dissolved in hot water (5 ml). The methanol extracts equivalent to 0.25 g of dry *E. bicyclis* was mixed into the agar solution and the mixture was cooled to make a block (20 x 20 x 0.7 mm). An agar block without any test sample in it was prepared as a reference.

The test samples using carrageenan (The Japan Fruit Products Co., Ltd.) or curdlan type polysaccharide 13140 (PS) (Takeda Yakuhin Kogyo Co., Ltd.) were made in a similar way.

3. For the Avicel plate method. The methanol extracts or sample solutions (40~80 μl) were applied with a micro-syringe onto the sample zone (ca. 25 ~ 30 mm in diameter, drawn with a compass) on an Avicel plate which was made as follows. Avicel SF (crystalline cellulose, Asahi Kasei Co., Ltd.) was coated on glass plates (5 or 10 x 20 cm) in 0.25 mm thickness in the conventional way and dried at 120°C for 1 hr.

Assay procedure for the feeding-stimulating activity.

1. Filter paper method. After sunset the sample paper and a reference one were set on the bottom of the test aquarium with lead sinkers on them. The supply of sea-water was stopped during the test. Next morning the feeding-stimulating activity of the sample was estimated by comparing the biting traces on the paper with those on the reference. It was not very easy to judge the activity by this method.

2. Polysaccharide method. Each sample block made using three kinds of polysaccharides as described above was placed in the test aquarium as in the case of the filter paper. The young abalone bit off more or less of both the sample block and the reference one. Not only was the sample preparation rather tedious but also judgement of the activity was much less clear than the filter paper method.

3. An Avicel plate method. The Avicel plate ready for the assay was set with the filter paper. Next morning by comparing the number of feeding traces left on the plate (Fig. 3), feeding-stimulating activity of each sample was judged as + + (the biting traces are observed almost all over the sample zone and the activity can be judged clearly; e.g. Frs. 5 and 6 in the plate A and Fr. 6 in the plate B in Fig. 3), + (much more biting traces are found in the sample zone than those outside in a unit area, but the judgement is not very clear; e.g. Fr. 7 in the plate B in Fig. 3), ± (a little more biting traces are observed in the sample zone than those outside and it is desirable to repeat the assay to judge the activity) and – (no or nearly the same number of the biting traces are left inside of the sample zone as those outside; e.g. Frs. in the plates C and D).
Extraction of seaweeds with methanol. Seven species of seaweeds, two of brown algae, *E. bicyclis* and *U. pinnatifida*, three of green ones, *Ulva sp.*, *Codium coaratum*, and *Codium latum*, and a red one, *Gelidium amansii* (Table I), which were harvested in May, 1982, in Shizuoka, were air-dried and soaked in methanol at room temperature for several days to obtain the methanol extracts.

Sephadex LH-20 column chromatography of the *E. bicyclis* extracts. The methanol extracts (ca. 350ml) of the dry *E. bicyclis* (35 g) were concentrated, applied onto a column (φ2 x 41 cm) of Sephadex LH-20 and eluted with methanol (each fraction, 10 g). Thirty microliters of each fraction (equivalent to 0.1 g of the dried alga, supposing that all the active principles contained in the algae had been eluted in a fraction) was applied onto a sample zone with a microsyringe. It is noteworthy that the MED (minimum effective dose) value of the crude methanol extracts of the alga is less than 13 mg equivalent (Table I). The sample amount should be enough though an active principle had been eluted into two or three fractions or two or three active principles had been present in the extracts.

RESULTS AND DISCUSSION

Careful observations of the feeding behavior of young abalones allowed us to consider that their feeding behaviors are based on stimulation of their olfaction or/and taste. Actually *Haliotis* spp. are known to have osfladium and taste buds.11

Many trials, including a Y-tube method,12 have resulted no simpler and more reliable bioassay method for attractants for young abalones than that recently developed by Harada *et al.*5

When a filter paper on which the methanol extracts of *E. bicyclis* (equivalent to 50 mg of the dried material) were absorbed was set in the test aquarium together with a reference one, the abalone bit off the paper with the extracts on it much more than the reference. They distinguished the paper with the extracts on it from the reference. It seemed promising to establish a bioassay method for feeding-stimulants for the young abalone. However, it was not very easy to estimate clearly their feeding preference from the biting traces left on the paper.

Next we tried to use polysaccharides for preparing test foods, but none of them gave clear-cut results. Finally our trial using Avicel plates proved fruitful.

Evaluation of the phagostimulant activity

The test Avicel plate with the sample on it was set in the experimental aquarium after sunset. Young abalones walked around in the tank, even on the test plate, searching for food (Fig. 2). We observed their interesting feeding behavior. They crawled on the test plate and bit off the Avicel at some intervals to leave a pair of their feeding traces here and there (Fig. 3). The abalone left mucus behind when they move and their moving traces are visible because the dust in the tank sticks to the mucus. The traces tell how often the abalones walked around searching for food. When they found a spot where their phagostimulant is absorbed, they bit off the Avicel of the sample zone (Fig. 3).

Even in the case when their food-searching activity is very high and a lot of their biting traces are left all over the plate (Fig. 3, plates A and C) the feeding preference could be judged.
Table I. Feeding-stimulating Activity of the Methanol Extracts of Algae on Young Abalone, Haliotis discus

<table>
<thead>
<tr>
<th>Name of algae</th>
<th>MED value$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Chlorophyceae]</td>
<td></td>
</tr>
<tr>
<td>Ulva sp.$^a$ &quot;Aosa&quot;</td>
<td>13~25</td>
</tr>
<tr>
<td>Codiaeae</td>
<td></td>
</tr>
<tr>
<td>Codium coaratum Okamura &quot;Nezashimiri&quot;</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Codium latum Suringar &quot;Hiramiru&quot;</td>
<td>42~85</td>
</tr>
<tr>
<td>[Phaeophyceae]</td>
<td></td>
</tr>
<tr>
<td>Dictyotaceae</td>
<td></td>
</tr>
<tr>
<td>Padina arborescens Holmes &quot;Umiuchiwa&quot;</td>
<td>&lt;13</td>
</tr>
<tr>
<td>Laminariaceae</td>
<td></td>
</tr>
<tr>
<td>Eisenia bicyclis (Kjellman) Setchell &quot;Arame&quot;</td>
<td>13~25</td>
</tr>
<tr>
<td>Alariaceae</td>
<td></td>
</tr>
<tr>
<td>Undaria pinnatifida (Harvey) Springar &quot;Wakame&quot;</td>
<td>&lt;13</td>
</tr>
<tr>
<td>[Rhodophyceae]</td>
<td></td>
</tr>
<tr>
<td>Gelidaceae</td>
<td></td>
</tr>
<tr>
<td>Gelidium amansii (Lamouroux) Lamouroux &quot;Makusa&quot;</td>
<td>13~25</td>
</tr>
</tbody>
</table>

$^a$ Supposed to be U. pertusa Kjellman.
$^b$ Minimum effective dose, mg equivalent to the air-dried alga. These values are preliminary.

by comparing the number of the traces as described in MATERIALS AND METHODS. On the other hand we observed once in a while that no abalone approached the test plate at all, even though enough of their phagostimulants had been present on it. The test was carried out at least twice for each sample and estimation of the activity was made by confirming the reproducibility of the activity.

Application of this procedure allows us to evaluate the feeding preference of the young abalone by the MED value (Table I).

Application of the assay procedure on the extracts of algae

Methanol extracts of three species of Chlorophyceae algae, Ulva sp., C. caratatum, and C. latum, of three Phaeophyceae P. arborescens, E. bicyclis, and U. pinnatifida, and of a Rhodophyceae alga, G. amansii, were subjected to the assay (Table I). The young abalones showed their feeding preference on almost all the extracts to a large or small extent. U. pinnatifida and P. arborescens were the most active. Methanol extracts equivalent to less than 13 mg of the dry material of either alga for each sample zone was enough for the abalone to show their feeding preference. It is quite interesting that the phagostimulant activity of these algae are not out of accordance with the efficiency of the algae as their foods observed by Kikuchi et al.$^6$)

Application of the assay method to the fractions from the methanol extracts of E. bicyclis

A brown alga, E. bicyclis, is now usually used to grow young abalone in an aquarium up to optimum size (20~30mm in shell length) to be liberated into the sea to increase the number of abalones. The methanol extracts were subjected to Sephadex LH-20 column chromatography to give 20 fractions. As shown in Fig. 3 the activity is concentrated into Frs. 6 and 7. The MED value (25~50 mg equivalent) of Frs. 6 and 7 explains that all the active principles are concentrated in these fractions, and probably no synergist is present.

This shows that the new bioassay method to search for phagostimulants for young abalone is simple, reliable, and applicable to the isolation of feeding-stimulants for young abalone. This assay method is probably applicable for phagostimulants for other herbivorous gastropods. The possibility is now under investigation.

Isolation and structure elucidation of the phagostimulants present in some of the algae tested is in progress now. These chemical studies on the feeding behavior of marine invertebrates will give us some clue to understand of their feeding behavior and will be useful to develop more efficient artificial diets for the young abalone.

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