Short Paper

Inducing substance for abalone larval metamorphosis from the crustose coralline alga *Hydrolithon samoense*

KIYOTAKE SUENAGA,1 HIDENARI HORI,1 HITOSHI ISHIDA,1 HARUO NUKAYA,1 RODNEY D. ROBERTS2 AND KUNIRO TSUJI1*

1School of Pharmaceutical Science, University of Shizuoka, Shizuoka 422-8526, Japan and
2Cawthron Institute, Nelson, New Zealand


Abalone larvae swim for several days before they metamorphose into a miniature adult organism. Crustose coralline algae (CCA) induce substratum-specific metamorphosis of abalone larvae. The metamorphosis is triggered by chemical substances, such as γ-aminobutyric acid (GABA)1 and thyroid hormones,2 but these substances have not been found as free compounds in CCA. Morse and Morse studied settlement-inducing substances from CCA and suggested that settlement was controlled by precursors or metabolites of phycoerythrin structurally related to GABA.3 However, the active substances in the algae remained unspecified.4 Seki et al. reported that abalone larval metamorphosis was induced by dibromomethane produced from algal surfaces.5 The chemical structure of CH₂Br₂ is quite different from that of GABA. The present study on inducing substances in CCA focused on low-molecular weight compounds, and resulted in the isolation of δ-aminovaleric acid (5-AVA) as an inducer.

Larvae of two species of abalone *Haliotis discus discus* and *Haliotis discus hannai* were supplied by Shizuoka Prefectural Sea Farming Center, Shizuoka Prefectural Thermal Effluent Utilization Research Center, and Yamagata Prefectural Sea Farming Center. Three or 4 days after fertilization at 18–20°C, the veliger larvae were transported to the University of Shizuoka. Assays were performed by slight modifications of the procedure described previously.6 Swimming larvae, which were competent to metamorphose, were placed in 24-well tissue culture dishes at densities of 20–40/well. Assays were performed in the dark at 19 ± 1°C in 2.0 mL sterilized natural seawater containing the tested samples and 150 μg/mL each of potassium penicillin G and streptomycin sulfate (Meiji Seika Kaisha Ltd, Tokyo, Japan). Two controls were included, one containing only seawater (negative) and the other 1 mM baclofen (positive), a potent GABA₄ receptor agonist.6 The percentage of metamorphosis in each well was determined at 96 h with the use of a microscope at 40× magnification. The assays were conducted in quadruplicate, and the results were shown as the mean value of four wells.

Stones covered with the CCA *Hydrolithon samoense* were collected at Osezaki (Shizuoka Prefecture, Japan). Algae-covered stones (estimated weight of wet algae, 300 g) were directly extracted with distilled water at 4°C for 2 days. After filtering, the filtrate was lyophilized to give powdered extract (11 g). The extract was separated by a series of chromatographic methods (Fig. 1) to give an active fraction (fr-1-2-3, 20 mg). The conditions for chromatography were as follows: (i) Diaion HP-20, solvent: H₂O → 50% aqueous methanol → methanol; (ii) activated charcoal, solvent: H₂O → 50% aqueous ethanol → 50% aqueous ethanol containing 1% acetic acid; (iii) Amberlite CG-50 (H⁺ form; Rohm and Haas Co., Philadelphia, PA, USA), solvent: H₂O → 0.1 M acetic acid → 1 M acetic acid. The active fraction was further purified to give δ-aminovaleric acid (5-AVA, fr-1-2-3-6-2, 3.2 mg) as an active substance. 5-AVA was also contained in

*Corresponding author: Tel: 81-54-264-5625. Fax: 81-54-263-4884. Email: tsuji@u-shizuoka-ken.ac.jp Received 17 June 2002. Accepted 21 October 2002.
the active fraction (fr-1-2-3-6-1), the activity of which corresponded to the 5-AVA content of the fraction. The conditions for chromatography were as follows: (iv) CM Sephadex C-25 (Amersham Pharmacia Biotech, Piscataway, NJ, USA), solvent: 0.1 M acetic acid; (v) HPLC, column: ODS (ϕ 10 × 250 mm, YMC Co., Ltd, Kyoto, Japan), solvent: 0.1% trifluoroacetic acid (TFA) → 30% aqueous methanol containing 0.1% TFA. Structural determination was performed by 1H and 13C nuclear magnetic resonance and fast atom bombardment mass spectrometry (FABMS). Separation of other active fractions (fr-2, fr 1-1, and fr 1-2-1) is currently in progress.

The settlement-inducing activity of commercially available 5-AVA has been tested previously for abalone larvae. 5-AVA induced attachment in *Halitos rufescense* at 1 mM, attachment without metamorphosis in *Haliotis virginea* at 1 mM, and attachment and metamorphosis of *Haliotis iris* at 100 μM but not 1 μM (R. Roberts, unpubl. data, 1999). In the present study (Table 1), commercially available 5-AVA exhibited inducing activity at 5 μg/mL (43 μM), which was comparable to the activity of the compound obtained from *Hydrolithon samoense* (fraction 1-2-3-6-2 in Fig. 1).

In the next experiment, quantitative analysis of GABA and 5-AVA concentrations in the algal extract was conducted by Marfey's method.6 The algae extract was reacted with Marfey's reagent, and the reaction mixture was analyzed by using reversed-phase HPLC [column: YMC ODS-AM, φ 4.6 × 250 mm, flow rate: 1 mL/min, solvent: methanol-0.02 M aqueous sodium acetate (pH 4) 60 : 40, detection: UV340 nm]. The retention time of derived authentic samples was as follows: GABA 12 min, 5-AVA 14 min. The 5-AVA component of the algal extract was 0.072%, which corresponded to ~10% of the total activity of the algal extract. In addition to the compound purified, metamorphosis-inducing activity was present in three additional fractions (Fig. 1, fr-1-1, fr-1-2-1, and fr-2), confirming previous speculation that CCA may contain multiple inducer compounds.9 In some cases, the unexplored active fractions were chromatographically distant from the active fraction pursued in the present study, suggesting that the various inducers in *Hydrolithon samoense* may represent quite different chemical structures. The active substances seemed to be very polar and basic low-molecular weight compounds based on chromatographic properties. Further studies on the remaining active substances are now underway. The potent inducer, GABA, was not detected in the extract of *Hydrolithon samoense*. Similarly, GABA was not detected in extracts of CCA by Kaspar and Mountfort10 and early reports of GABA in CCA extracts,1 in fact, referred to larger GABA-mimetic molecules.4

In conclusion, we isolated δ-aminovaleric acid (5-AVA) as an inducing substance for the abalone larval metamorphosis from CCA, *Hydrolithon samoense*. Although Morse reported

![Fig. 1 Separation scheme for the metamorphosis-inducing fraction from the alga *Hydrolithon samoense*. The weight of each fraction is in parentheses. The inducing activity for abalone larval metamorphosis of each fraction is in brackets [dose (μg/mL), the rate of metamorphosis (%), mean ± SD]). Fractions with substantial activity are in boxes.](image)

<table>
<thead>
<tr>
<th>Sample dose (μM)</th>
<th>Rate of metamorphosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control (sea water)</td>
<td>1.0 ± 1.9</td>
</tr>
<tr>
<td>Positive control (baclofen)</td>
<td>81.9 ± 15.6</td>
</tr>
<tr>
<td>δ-Aminovaleric acid</td>
<td>1.7 ± 1.9</td>
</tr>
<tr>
<td>6.8</td>
<td>3.8 ± 7.5</td>
</tr>
<tr>
<td>17</td>
<td>23.8 ± 7.6</td>
</tr>
<tr>
<td>43</td>
<td>69.6 ± 12.8</td>
</tr>
<tr>
<td>170</td>
<td>72.5 ± 7.1</td>
</tr>
</tbody>
</table>

1Values are the mean ± SD of quadruplicate experiments.
that 5-AVA exhibited inducing activity based on testing of various pure compounds, such as amino acids, there is no previous report that 5-AVA is a constituent of CCA. Our studies confirmed the presence of 5-AVA in the alga *Hydrolithon samoense*, and suggest that this compound may be an ecologically relevant inducer of abalone metamorphosis. The chemical structure of 5-AVA is very similar to that of δ-aminolevulinic acid, a biosynthetic precursor of tetrapyrrrole pigments, such as phycoerythrin found in CCA. 5-AVA is a GABA antagonist and antagonizes pharmacological actions of baclofen in the central nervous system. 5-AVA was isolated from the anaerobic bacterium *Clostridium bifermentans*. There are no reports on the existence of 5-AVA in algae, but related compounds, such as gigartinine (L-α-amino-δ-(guanylureido)-n-valeric acid), were isolated from some red algae. Further studies on the inducing substances are currently in progress.

We thank Messrs Kenichi Satake (Shizuoka Prefectural Thermal Effluent Utilization Research Center), Hiroyuki Kawakami (Yamagata Prefectural Sea Farming Center), and Takayuki Hanai (Shizuoka Prefectural Sea Farming Center) for donation of abalone larvae and their helpful advice and discussions. We thank Professor Shinpei Ueno (Tokai University) and Dr Masasuke Baba (Marine Ecology Research Institute) for identification of the alga.

REFERENCES


