Communications to the Editor

[Chem. Pharm. Bull. 31(10)3771—3774(1983)]

ISOLATION FROM HUMUS AND IDENTIFICATION OF TWO GROWTH PROMOTERS, ADENOSINE AND 2'-DEOXYADENOSINE, EFFECTIVE IN CULTURING THE DIATOM PHAEODACTYLUM TRICORNUTUM

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Two promoters of growth in the diatom Phaeodactylum tricornutum were isolated from humus and identified as adenosine and 2'-deoxyadenosine, respectively, both of which are effective even in the extremely low concentration of 10^{-4}nM.

KEYWORDS ——— growth promoter; diatom; Phaeodactylum tricornutum; adenosine; 2'-deoxyadenosine; icosapentaenoic acid; EPA; humus; matured compost

It was reported that the component fatty-acid mixture of lipids contained in the diatom Phaeodactylum tricornutum was similar to that of fish oil, in which highly unsaturated fatty acids predominated.[1] In these acids, (all-Z)-5,8,11,14,17-icosapentaenoic acid (EPA) has potent anti-thrombotic and anti-atherosclerotic effects and may reduce the incidence of thrombotic cardiovascular disorders.[2] P. tricornutum is expected to be an excellent source of this medically important fatty acid. Therefore, we have investigated how to establish an efficient mass culturing method of P. tricornutum and during the course of this study we have succeeded in isolating two growth promoters from humus and identifing them as adenosine and 2'-deoxyadenosine, respectively.

Soil extract have long been known to contain various growth promoters on phytoplanktons, the active compounds of which have been attributed to humic substances, vitamins, phytohormones and chelating agents.[3] The real active compounds, however have not yet been characterized. We studied growth promoting activity of the soil extract and humus extract, respectively, affecting P. tricornutum cultured in the revised Haxo-Sweeney culture medium (Fig. 1), and found that the aqueous extract of humus (HE), a matured compost of rice straws prepared only with water, showed stronger activity than that of the aqueous extract of soil (Fig. 2). Therefore, we have isolated and identified growth promoters contained in the HE.

The method of bioassaying growth regulating activity is outlined in Fig. 1. A batch of 12 cylindrical test tubes (6 x 50 cm) containing 800 ml of the revised Haxo-Sweeney culture medium was immersed in one side of a water thermostat (20 °C). Each two test tubes were separated from the others by partition and were illuminated laterally with a 150 W incandescent lamp, respectively. Their distance was adjusted beforehand so the light intensity at the center of each test tube was

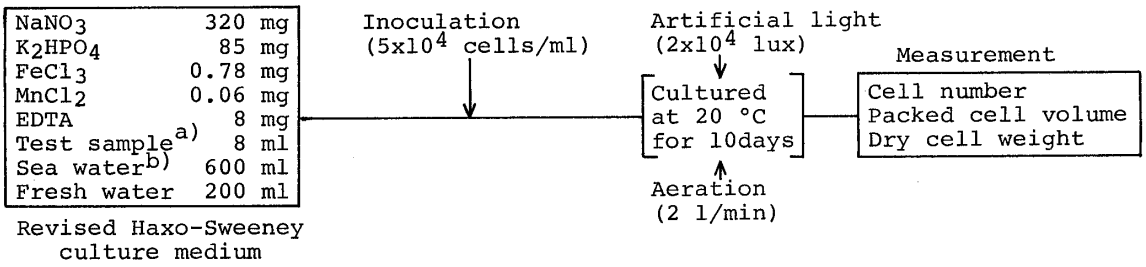

Fig. 1. Outline of Bioassay Method of Growth Regulating Activity on the Diatom Phaeodactylum tricornutum

a); Each test sample was dissolved with 8 ml of fresh water. b); Only sea water obtained from the surface layer of the Pacific Ocean and brought by the Umitaka-maru of Tokyo University of Fisheries was used. The pH of the medium was 8.0 before inoculation and 8.4-9.0 after culturing for 10 days. Cell number (5-7 x 10^7 cells/ml), packed cell volume (6-8 ml) and dry cell weight (1.0-1.5 g) were obtained in the control cultures which were cultured for 10 days after addition of only fresh water (8 ml).

20,000 lux. An aeration tube plugged with cotton was inserted into the center of each test tube. Aeration throughout the study was carried out with air passing through double cotton tubes sterilized at 120 °C and no contamination has ever occurred even without sterilization of other parts of the apparatus. After addition of each test sample to each test tube, a unialgal culture of P. tricornutum, cultured in a glass tube (3 x 20 cm) containing 50 ml of the revised Haxo-Sweeney culture medium under artificial light (3,000 lux) without aeration for 10-15 days (the cell number at this stage is ordinarily 3 x 10^6 cells/ml), was inoculated into each test tube. The number of cells in the inoculating culture was measured beforehand with a Coulter counter (Industrial type) and a Thoma hemacytometer, and the initial cell number of each test tube was adjusted to 50,000 cells/ml. After culturing for 10 days, the total cell number, packed cell volume and especially dry cell weight, the most reliable comparative parameter, were measured and compared with the control culture.

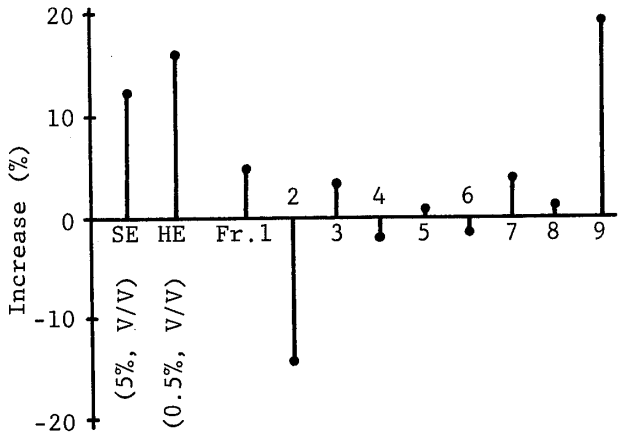

Fig. 2. Growth Regulating Activity of Soil Extract (SE), Humus Extract (HE) and Fractions of the First Column Chromatography over Sephadex G15

The numbers of the increase indicate the percentage increase of dry cell weight compared with that of the control.

In order to prepare the HE, the humus (1.5 kg) was heated with 1.5 l of water on a boiling water bath for 1 h. After cooling, the mixture was filtered through a thin talcum-layered filter paper, then a Millipore filter (0.8 μ) to give 1.3 l of the filtrate which was concentrated in vacuo to one-tenth volume and kept in a freezer until used.

The concentrated aqueous solution of HE corresponding to 220 g of the humus was centrifuged to remove insoluble materials. The supernatant was treated by column chromatography over Sephadex G15 (16 x 847 mm) eluted with aqueous ammonium acetate ($AcONH_4$) buffer solution (0.03 M, pH 6.7) and the eluate was divided into 9 fractions. The significant growth promoting activity was

found in the 9th fraction (eluate of 260 to 360 ml) and the growth inhibiting effect in the 2nd fraction (85 to 135 ml) (Fig. 2). The 9th fraction was further purified by preparative high-performance liquid chromatography (HPLC) to give 13 fractions (A to M) as shown in Fig. 3. The 3rd and 5th fractions, each corresponding to single peaks which were designated compounds C and E, respectively, showed the growth promoting activity.

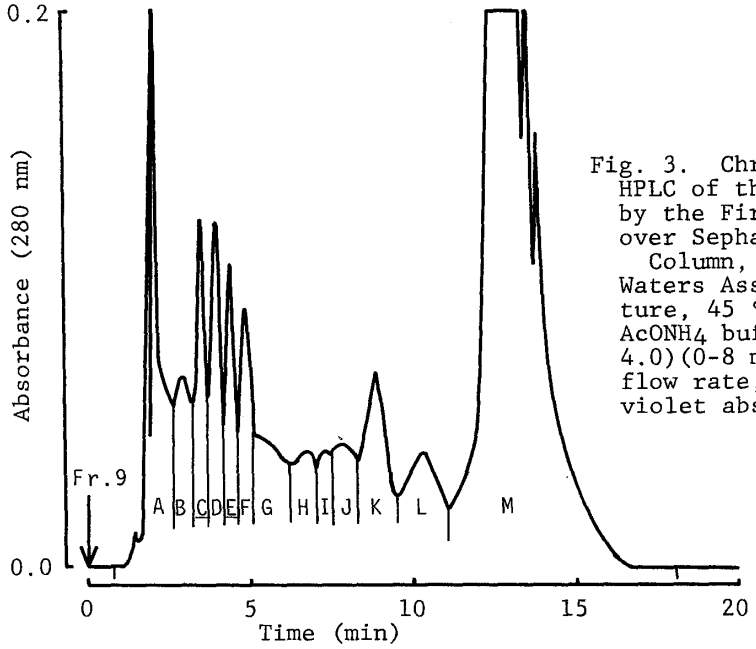

Fig. 3. Chromatogram of Preparative HPLC of the 9th Fraction Obtained by the First Column Chromatography over Sephadex G15
Column, μBondapak C_{18} (4mm x 30cm, Waters Associates); column temperature, 45 °C; mobile phase, 5% CH_3CN-$AcONH_4$ buffer solution (0.01 M, pH 4.0)(0-8 min) and methanol (8-18 min); flow rate, 2 ml/min; detector, ultra violet absorption at 280 nm.

In order to isolate enough material for structural characterization of compounds C and E, the aqueous solution (6.1 l, 56.9 g if lyophilized) of the HE obtained from 4.7 kg of the humus was treated in the following way, monitoring by HPLC analysis. After being adjusted to pH 3.5 with acetic acid and centrifuged, the supernatant was passed through a SP Sephadex column (44 x 658 mm) which had been equilibrated with $AcONH_4$ buffer solution (0.01 M, pH 3.5). The column was washed with 250 ml of $AcONH_4$ buffer solution (0.01 M, pH 4.0), then with 400 ml of $AcONH_4$ buffer solution (0.02 M, pH 6.5), connected with a fraction collector and eluted with the latter solution to get 40 fractions (20 ml/fraction). Compounds C and E were found in fractions 29-35 by HPLC analysis. A combined mixture (238 mg) of fractions 29-35 was fractionated by column chromatography over Sephadex G15 (10 x 535 mm) eluted with $AcONH_4$ buffer solution (0.03 M, pH 6.5). The eluate of 50 to 92 ml containing compounds C and E was lyophilized to give 27 mg of the mixture which was further purified by column chromatography over Sephadex G10 (10 x 564 mm) eluted with $AcONH_4$ buffer solution (0.03 M, pH 6.5). Compounds C and E were found in the eluate of 65 to 93 ml which was lyophilized (1.2 mg) and applied to a preparative HPLC [column, Radial-pak C_{18} (8 mm x 10 cm, Waters Associates); room temperature; mobile phase, 1% CH_3CN-$AcONH_4$ buffer solution (0.01 M, pH 4.0); flow rate, 1 ml/min; detector, ultra violet (UV) absorption at 254 and 280 nm] to isolate compounds C (208 μg) and E (184 μg), respectively.

The UV spectra [compound C: $\lambda_{max}^{H_2O}$ 257 nm(pH 2), 260(6) and 260(12); $\lambda_{min}^{H_2O}$ 230(2), 227(6), and 230(12); A280/A260 0.215(2), 0.149(6) and 0.161(12). compound E: $\lambda_{max}^{H_2O}$ 258(2), 260(6) and 260(12); $\lambda_{min}^{H_2O}$ 228(2), 226(6) and 230(12); A280/A260 0.221(2), 0.152(6) and 0.159(12)], mass spectra [compound C: m/z 267(M^+), 250, 237, 178, 164,

148, 136, 135(base peak), 119 and 108. compound E: m/z 251(M^+), 234, 221, 164, 162, 136, 135(base peak), 119 and 108], nuclear magnetic resonance (NMR) spectra [270 MHz, DMSO-d_6, δ. compound C: 3.56(1H, dd, J=12.2 and 3.6 Hz), 3.67(1H, dd, J=12.2 and 3.6), 3.96(1H, dd, J=3.6 and 4.1), 4.14(1H, t, J=4.1 and 5.4), 4.60(1H, t, J=5.4 and 5.6), 5.87(1H, d, J=5.6), 8.15(1H, s) and 8.37(1H, s). compound E: 2.25 (1H, m), 2.72(1H, m), 3.50(1H, dd, J=11.9 and 4.0), 3.61(1H, dd, J=11.9 and 4.0), 3.88(1H, m), 4.40(1H, m), 6.33(1H, dd, J=6.3 and 7.6), 8.12(1H, s) and 8.32(1H, s)], and the various chromatographic behaviours suggested that compounds C and E might be adenosine and 2'-deoxyadenosine, respectively. They were identified with the authentic samples by comparison of the UV, mass and NMR spectra and by HPLC.

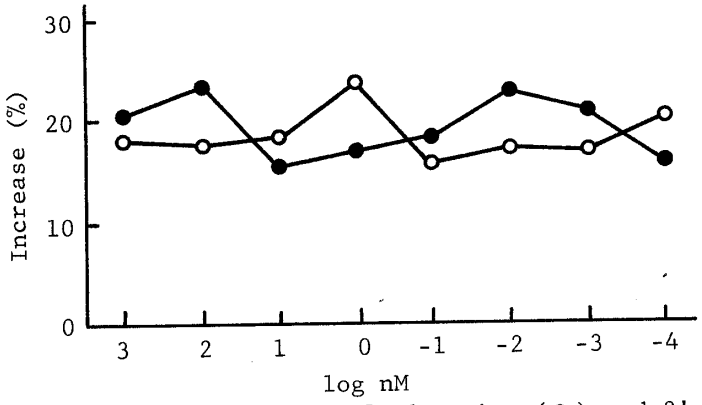

Fig. 4. Growth Promoting Activity of Adenosine (●) and 2'-Deoxyadenosine (○)
The numbers of the increase indicate the percentage increase of dry cell weight compared with that of the control.

Both adenosine and 2'-deoxyadenosine promoted growth in P. tricornutum even in the extremely low concentration of 10^{-4} nM. The potency is not concentration-dependent and the increase in dry cell weight of 15-25% was obtained at each concentration more than 10^{-4} nM as shown in Fig. 4.

Yeastolate, yeast extract, trypticase, digests of animal tissues and decomposed matter of pearl oyster's faeces have been reported as natural organic materials other than soil extract which promote the growth of phytoplanktons.[3] It is suggested from the present finding that some active components contained in these materials may be adenosine and/or 2'-deoxyadenosine. It was also reported that nucleic bases showed the growth promoting effect in Dinophyceae.[3]

Now in progress are the isolation of the growth inhibiting compound and investigation of the compounds related to adenosine and 2'-deoxyadenosine having activities that promote growth in P. tricornutum.

ACKNOWLEDGEMENT We thank Professor H. Aruga of Tokyo University of Fisheries for his generous supply of sea water. This research was supported by a Grant-in-Aid from Nishiyama Corporation.

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(Received August 4, 1983)