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Functional Sulfur Amino Acid Production and Seawater Remediation System by Sterile *Ulva* sp. (Chlorophyta)

Shin Hirayama,*[,1] Masashi Miyasaka,[2] Hideomi Amano,[3] Yoshito Kumagai,[4] Nobuhiro Shimojo,[4] Teruyoshi Yanagita,[5] and Yoshiro Okami[6]

[1]Advanced Technology Research Center, Mitsubishi Heavy Industries, Ltd., 1-8-1, Sachiura, Kanazawa-Ku, Yokohama 236-8515, Japan, E-mail: shin_hirayama@mhi.co.jp; [2]Yokohama Dockyard & Machinery Works, Mitsubishi Heavy Industries, Ltd., 12, Nishiki-Cho, Naka-Ku, Yokohama 231-8715, Japan; [3]Faculty of Bioresources, Mie University, Tsu, Mie 514-8507, Japan; [4]Institute of Community Medicine, University of Tsukuba, Tsukuba, Ibaraki, 305-8575, Japan; [5]Department of Applied Biological Sciences, Saga University, Saga 840-8502, Japan; and [6]Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141-0021, Japan

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Abstract

Sterile *Ulva*, which is a macroalga, has the potential to grow stably; therefore, this seaweed is expected to be an efficient resource of functional food containing various nutrients such as sulfur amino acids, proteins, carbohydrates, and minerals. *Ulva lactuca* was selected from the "Marine Park" in Tokyo Bay, and its growth rate (g-dry/[m^2·d]) was measured using model reactors located on the land or on the surface of the sea at Yokohama. The growth rate of *U. lactuca* was recorded to be approx 20 g-dry/(m^2·d), which is estimated to be 10 times greater than that in a natural field in the Marine Park. In addition, this growth rate was higher than that of conventional crops such as corn and rice on a farm or paddy. These data led us to newly design and propose a floating type of labor-efficient *U. lactuca* production system. D-Cysteinolic acid, which is included in *U. lactuca* as a major sulfur amino acid, inhibited the Fenton reaction, resulting in suppression of hydroxyl radical production and singlet oxygen. Addition of the sulfur amino acid (1 μ*M*) to HepG2 cells markedly decreased the intracellular triglyceride level.

*Author to whom all correspondence and reprint requests should be addressed.

Hence, this proposed facility also has the potential for industrial production of a valuable resource for the primary prevention of lifestyle-related diseases using enriched or eutrophied seawater.

Index Entries: D-Cysteinolic acid; reactive oxygen; triglyceride; *Ulva lactuca*; seawater.

Introduction

Since the 1980s, a few physiologic activities of D-cysteinolic acid, including use as a platelet antiaggregant, have been reported by using fresh sardines showing a slight content of D-cysteinolic acid *(1,2)*. Because D-cysteinolic acid content of fresh sardine is very low, physiological research for D-cysteinolic acid was difficult. For this reason, more physiologic activities of D-cysteinolic acid could not be examined in detail. A high content of D-cysteinolic acid was reported in *Ulva* sp. *(3)*; therefore, a mutant of *Ulva lactuca*, which is able to grow under cultivation conditions in any season, is expected to be useful for efficient D-cysteinolic acid production *(4–7)*. In addition, because sterile *Ulva* sp. has been examined from the viewpoint of biomass material in eutrophied seawater *(8)*, it is expected to have valuable multiutilities when other physiologic activities including D-cysteinolic acid are discovered *(9,10)*.

On the other hand, along with a change in dietary habits including assimilation of oily foods, and an aging society, lifestyle-related diseases such as hypertriglyceridemia, hyperlipemia, and obesity have increased in Japan *(11)*. Under these circumstances, functional foods must be effective in the primary prevention of such diseases. Seaweeds including *Ulva* sp. are similar to Japanese food and have long been used as regional food. From the viewpoint of primary prevention of lifestyle-related diseases, D-cysteinolic acid, the major free amino acid in *Ulva* sp., was thought to be significant for its biologic function.

We report here that D-cysteinolic acid has a reactive oxygen scavenging effect as well as an antihypertriglyceridemic activity. We also propose a D-cysteinolic acid production system using a *U. lactuca* cultivation facility located in an enriched seawater area.

Materials and Methods

Isolation of D-Cysteinolic Acid

U. lactuca from the "Marine Park" in Yokohama was used for the purification of D-cysteinolic acid. D-Cysteinolic acid was purified as follows *(3)*: The air-dried specimen was crushed and extracted three times with 17 vol of 60% ethanol at 70°C. The extracts were slightly concentrated and defatted with diethyl ether. After the removal of diethyl ether, the aqueous solution was applied to and passed through a Dowex 50W × 8 column in H^+ form to remove the major amino acids except for strongly acidic amino acids. The effluent and washings were combined and passed through a

Dowex 2 × 8 column in OH^- form. After washing with water, the absorbed fraction was eluted with 4% acetic acid.

The effluent positive to ninhydrin reagent was recovered and concentrated to remove excess acetic acid. The concentrated residue was dissolved in water and passed through a small Dowex 2 × 8 column in OH^- form. The effluent positive to ninhydrin reagent was collected and then concentrated to a dried syrup. The syrup was dissolved in aqueous ethanol and kept in a cool place for crystallization. Crystallization was carried out twice with aqueous ethanol.

Identification of D-Cysteinolic Acid

The obtained crystals were analyzed by liquid chromatography mass spectrometry (LC-MS) with a mass-to-charge ratio (*m/z*) range of 60–300 at a scan duration of 0.45 s. High-performance liquid chromatography equipped with an L-column ODS was done with an HP1100 system (Hewlett Packard) using 30 m*M* CH_3COONH_4 (pH 7.0) containing 20% (v/v) CH_3CN as the eluent at a flow rate of 0.5 mL/min. Electrospray ionization was performed as the mass spectrometry with a Quattro-II system (Micromass) using negative polarity.

Reactive Oxygen Analysis

The samples contained riboflavin, D-cysteinolic acid, or taurine, and 2,2,6,6-tetramethyl-4-piperidone (TMPD) in 0.2 *M* phosphate buffer (pH 7.8). Individual samples were ultraviolet (UV) irradiated from a UV spot system (L2859-01; Hamamatsu, Shizuoka, Japan) for 2 min, and 4.8 mW/cm^2 was determined by the 365-nm sensor (UIT-102; Ushio, Tokyo, Japan) *(12)*. 2,2,6,6-Tetramethyl-4-piperidone-1-oxyl was used as a standard for the photooxidation product of a singlet oxygen acceptor, TMPD, by riboflavin photosensitization and was detected by electron spin resonance (ESR) spectrometry.

Superoxide was generated by 1.25 m*M* hypoxanthine and 0.1 U/mL of xanthine oxidase in 1.4 *M* dimethylsulfoxide containing 90 m*M* 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO).

Hydroxyl radicals were generated by a Fenton reaction system. The reaction mixture contained 38 μ*M* ferrous sulfate, 19 μ*M* diethylenetriaminepentaacetic acid, 9 m*M* DMPO and 0.38 m*M* H_2O_2. In addition, a spin-trapping study was also conducted in the UV-lighting system containing 0.5 m*M* H_2O_2 using 150 m*M* DMPO *(12)*.

These active oxygens were analyzed using an ESR spectrometer, JEOL Model RE-1X, having an aqueous quartz flat cell. The Mn^{2+} cation fixed in the ESR cavity was used as an internal standard to calculate the relative amounts of ESR signal intensity.

HepG2 Cell Culture

HepG2 cells were cultivated in Dulbecco's modified Eagle's medium (GIBCO, Santa Clara, CA) supplemented with 10% fetal calf serum, 1×10^5 U/L

of penicillin, and 100 mg/L of streptomycin at 37°C in an atmosphere containing 95% air and 5% CO_2 *(11)*.

Determination of Lipids

Total lipids were extracted by Bligh and Dyer's method and purified as reported previously *(11)*. The concentrations of triacylglycerol (or triglyceride) and total cholesterol were measured with commercial kits supplied by Wako Pure Chemical (Osaka, Japan).

Measurement of U. Lactuca *Growth Rate*

Three hundred pieces of *U. lactuca* cut on the same scale in a circular shape were placed into the culture space on which the light intensity at the surface and the bottom was adjusted to a ratio of 100:1 as the starting condition.

The relative evaluation rate of the *U. lactuca* was calculated from the diameter and its conversion coefficient of 10 random sheets harvested from the outdoor reactors every day; the resulting weight of the *U. lactuca* at the end of the culture was washed twice with deionized water and then dried on a ceramic cup of known weight in an oven at 110°C until a constant weight was reached. From this weight, the *Ulva* growth rate was calculated as the dry weight-g/(m^2·d).

Results and Discussion

Purification and Determination of D*-Cysteinolic Acid*

White needle-shaped crystals were obtained by the discussed purification steps, and LC-MS analysis showed a single peak, with a pseudomolecular ion of 154, and 80 estimated to be SO_3^-. Furthermore, the specific rotation $[\alpha]^{25}$ + 8.7 (in water) was closely identical to that of D-cysteinolic acid. These crystals were thought to be D-cysteinolic acid crystals (*see* Fig. 1) *(3)*.

However, because the sulfur amino acid taurine, similar to D-cysteinolic acid, was reported to have antioxidative physiologic activities *(13–17)*, these D-cysteinolic acids were used for physiologic activities such as reactive oxygen scavenging effects and suppression of lipid formation compared with taurine from the viewpoint of primary prevention of lifestyle-related diseases.

Scavenging Effect for Reactive Oxygen Species

D-Cysteinolic acid indicated a decrease in DMPO-OH adducts in the Fenton reaction system shown in Fig. 2, and its suppression was dependent on the concentration of D-cysteinolic acid. Taurine did not show the decreasing effect on DMPO-OH adducts in the Fenton reaction system (Fig. 2). To clarify the mechanism of this DMPO-OH inhibition, another hydroxyl radical production system by H_2O_2-UV was used instead of the Fenton reaction system. Because D-cysteinolic acid did not decrease the DMPO-OH adduct in the H_2O_2-UV system shown in Fig. 3, inhibition of the

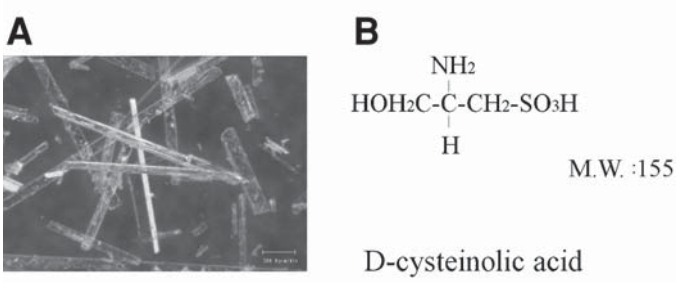

Fig. 1. D-cysteinolic acid. **(A)** D-Cysteinolic acid crystals; **(B)** chemical structure of D-cysteinolic acid.

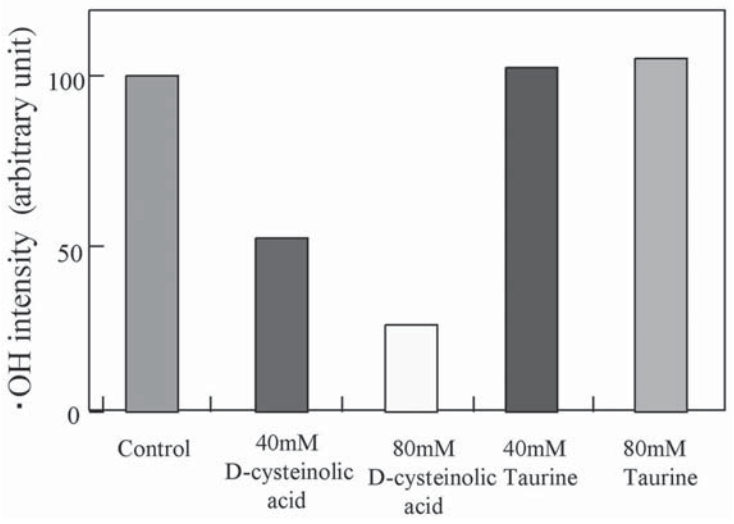

Fig. 2. Fenton reaction suppression activity of D-cysteinolic acid and taurine.

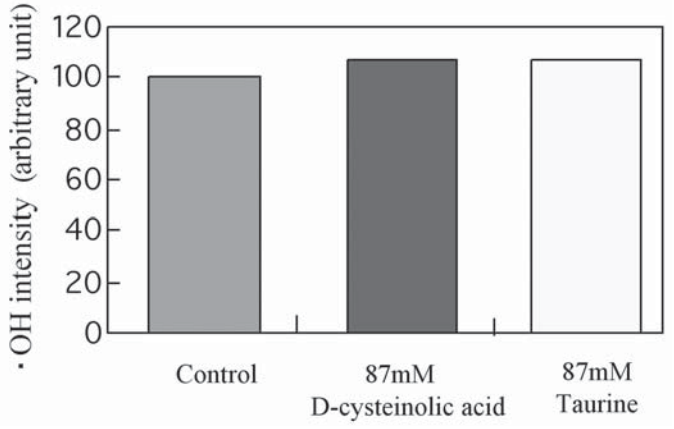

Fig. 3. Hydroxyl radical scavenging activity of D-cysteinolic acid and taurine under an H_2O_2-UV system.

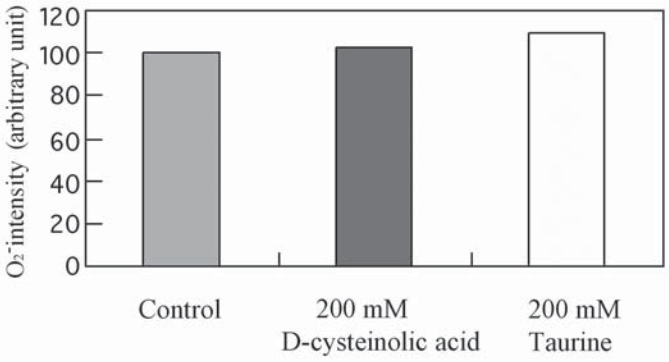

Fig. 4. Superoxide scavenging activity of D-cysteinolic acid and taurine.

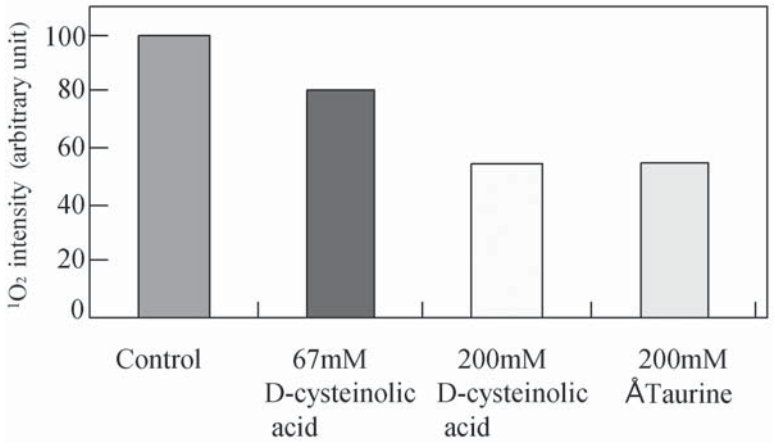

Fig. 5. Singlet oxygen quenching activity of D-cysteinolic acid and taurine.

DMPO-OH was owing to the Fenton reaction itself and was not thought to be owing to the scavenging of free hydroxyl radicals. While the inhibition mechanism is not clear at present, one of the mechanisms may be the chelating action of D-cysteinolic acid to Fe^{2+}, leading to inhibition of the Fenton reaction including attack from Fe^{2+} to H_2O_2. In addition, the Fenton reaction was thought to be a major contributor to hydroxyl radical production in biologic systems. Inhibition of the Fenton reaction by D-cysteinolic acid was thought to be important for in vivo antioxidation related to the prevention of hydroxyl radical production.

Superoxide from xanthin-xanthin oxidase was not suppressed by both D-cysteinolic acid and taurine (Fig. 4). Singlet oxygen was suppressed by both D-cysteinolic acid and taurine, and their activity was at almost the same level, as shown in Fig. 5.

These data show that D-cysteinolic acid has a much wider spectrum for reactive oxygen scavenging activity than does taurine.

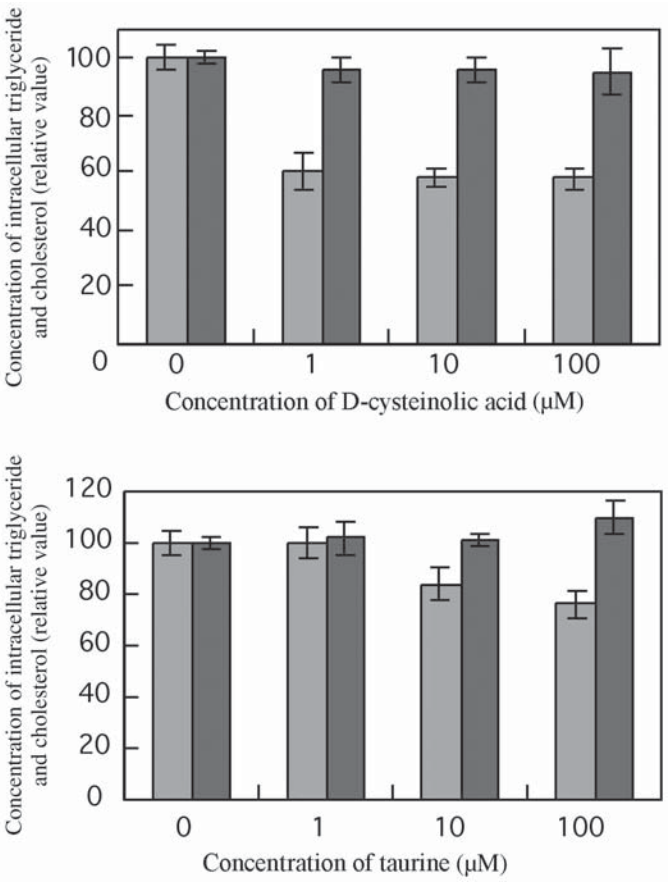

Fig. 6. Comparison of effect of D-cysteinolic acid and taurine on concentrations of triglyceride and cholesterol in HepG2 cells. Data are expressed as mean of four experiments. Light gray bars represent triglyceride; dark gray bars represent cholesterol.

Effect of Decreasing Lipid Formation

Because some sulfur compounds were effective for suppression of lipids such as triglyceride and cholesterol *(18,19)*, the effect of D-cysteinolic acid on lipid suppression compared with taurine was investigated using HepG2 cells that retained the human hepatogenic functions including lipid metabolism. These cells were cultured in a basal medium with D-cysteinolic acid or taurine to investigate their effect on lipid formation. Figure 6 shows that both D-cysteinolic acid and taurine had no effect on cholesterol; a low density of D-cysteinolic acid showed a decrease of approx 40% in the formation of intracellular triglyceride, and a high density of taurine showed a decrease of approx 20% in triglyceride. These data demonstrate that D-cysteinolic acid has a much stronger effect than taurine for suppression of the accumulation of triglyceride.

D-Cysteinolic acid as the main amino acid obtained from *Ulva* sp. has long been used as a regional food in Japan; therefore, D-cysteinolic acid was

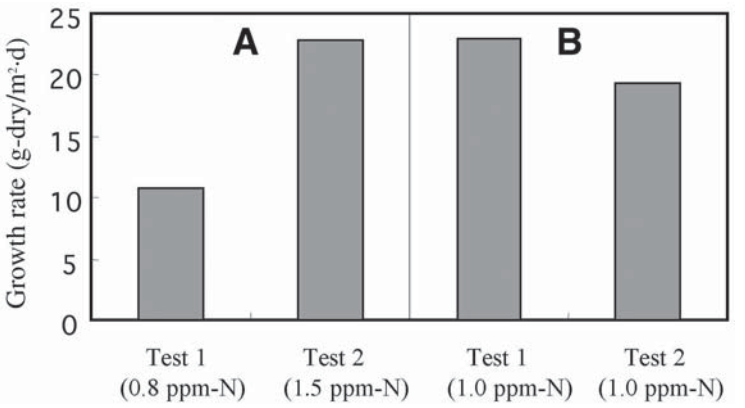

Fig. 7. Comparison of growth rate of *U. lactuca* in some model reactors: **(A)** settled on land under aeration at approx 0.8 or 1.5 ppm (mg/L) NO_3-N for 20 d; **(B)** floating reactor on sea surface under aeration at approx 1.0 ppm (mg/L) NO_3-N for 7 d in Tokyo Bay.

expected to be a safe medication for hypertriglyceridemia, hyperlipemia, and obesity, which cause the accumulation of triglyceride. Because D-cysteinolic acid was thought to be a remarkably functional compound for the primary prevention of such diseases, an effective D-cysteinolic acid production system using *Ulva* sp. was examined and is discussed next.

Growth Rate of U. Lactuca *in Culture Facility on the Sea*

Because the growth rate of *Ulva* for 10 or 20 d on the land was found to be 11–23 g-dry/(m^2·d) in an outdoor culture simulated on the surface of the sea (see Fig. 7A), a model culture plant was produced experimentally, and the growth rate of *U. lactuca* was measured in the model culture plant located on the sea surface at Yokohama. The growth rate was found to be approx 20 g-dry/(m^2·d) for 1 wk on the practical sea (*see* Fig. 7B). From the relationships between the carbon content of *U. lactuca* and the amount of CO_2 supplied in aeration as the carbon source to the *U. lactuca*, a 20 g-dry/(m^2·d) growth rate is thought to be a theoretical value for the reactor.

On the other hand, the growth rate of *Ulva* was estimated to be 1–2 g-dry/(m^2·d) on a natural field at the Marine Park in Yokohama *(20)*. Hence, approximately a 10-fold increase in the growth rate in the field is attainable by controlled conditions such as aeration in summer. In addition, this growth rate is higher than that of conventional crops such as corn, wheat, and rice in the temperate zone *(20,21)*. Consequently, the culture of *U. lactuca* is thought to be a means for effective biomass production. Because *U. lactuca* also contains approx 0.5% (w/w) D-cysteinolic acid, which is a higher content than other resources such as *Sardinops melanosticta* and *Asterina pectinifera (3)*, the cultivation of *U. lactuca* is expected to provide an effective means of D-cysteinolic acid production.

Because more than 10 g-dry/(m^2·d) of growth on the land was obtained with accumulation of both N and P in outdoor culture at an adjusted

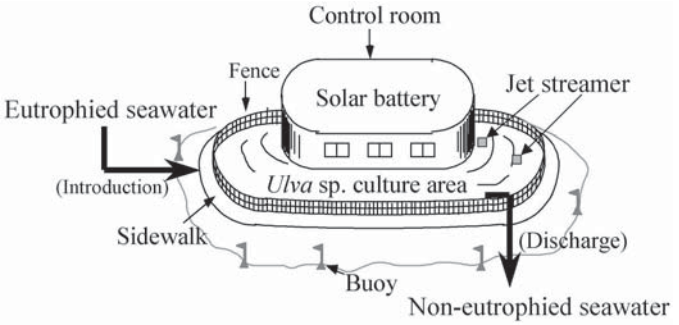

Fig. 8. Plan of floating model plant for *U. lactuca* production.

temperature of approx 25°C in winter (see Fig. 7A) *(20)*, the production of *U. lactuca* must be done year round even when the culture temperature is controlled by heating or using warm seawater in winter. Future studies over a long period need to be conducted. *Ulva* cultivation facilities are thought to be important for a valuable production system with environmental accommodation.

Concept for Production Facilities of Ulva *sp.*

Because of the discussed advantages of *Ulva* sp., we newly designed and proposed a floating type of a *U. lactuca* production system, as shown in Fig. 8. It is a floating body similar to a raceway on the eutrophied sea. This facility mainly consists of a stirring apparatus for supplies of both CO_2 and sunlight and a harvesting apparatus for the effective production of *U. lactuca*. Jet streamers are used as the stirring apparatus for energy conservation. Electric power is supplied to the facilities by a solar battery and a power plant from the land. Because *U. lactuca* can be used as a resource of D-cysteinolic acid, food material, or an additive to feed, this macroalga has enormous potential. Hence, this facility has a potential not only for D-cysteinolic acid production for primary prevention of lifestyle-related diseases but also for biomass production using enriched seawater. In addition, this system has potential for remediation of coastal eutrophied seawater.

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