Biochemical Studies on the Action Mechanisms of Bioactive Substances Discovered from Various Marine Algal Species

海洋微細藻類及び大型藻類に存在する多様な生理活性物質の作用機構に関する生化学的研究

2019年5月
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CHAPTER I

General Introduction

Our planet's surface is covered more than 70% by the oceans and numerous beneficial marine products have been found including polysaccharides, essential minerals and vitamins, antioxidant, enzymes, proteins and peptides. These findings suggest that the ocean supplies increasingly important source of bioactive material with enormous potential. Marine algae, diverse group of aquatic organisms that have the ability to conduct photosynthesis, provide both benefit and harm for marine ecosystems. Seaweeds have been used as foods, animal feeds, fertiliser and as sources of traditional medicine in many Asian civilisations since ancient times. Seaweeds are excellent dietary sources of vitamins, proteins, carbohydrates and trace elements, but also nutraceutical potentials like antioxidant, antimutagenic, anticoagulant, anticancerous and antibacterial activity.[1] Hence, seaweeds can be considered as promising plants forming one of the important marine living resources of high nutritional value. On the other hand, harmful algal blooms (HABs) are a serious environmental issue. Concurrently with global warming, there have been a worldwide increase in the frequency, magnitude, and duration of HABs.[2,3] Therefore, their threat to aquaculture, fisheries, and public health in coastal waters throughout the world has increased.[4]

Heterocapsa circularisquama is a toxic dinoflagellate that has been causing mass mortality of bivalves in the coastal areas of western Japan since 1988.[5] Harmful algal blooms (HABs) of *H. circularisquama* have been rapidly increasing since early 1990. A characteristic feature of this dinoflagellate is that it is known to be highly toxic to bivalves such as the pearl oyster (*Pinctada fucata*), short-necked clam (*Ruditapes philippinarum*), and oyster (*Crassostrea gigas*). Harmful effects on wild and cultured finfish, other marine vertebrates, and public health have not been reported so far.[6,7] Pearl oysters exposed to $>10^6$ *H. circularisquama* cells L^{-1} in laboratory exposures immediately contracted their mantles and closed their valves, became paralysed, and then eventually died.[8] These symptoms closely resembled those of previous field observations.[9] In addition, paralytic shellfish poisoning (PSP) and diarrheic shellfish poisoning (DSP) toxins in the *H. circularisquama* cells have not been detected by direct HPLC analysis yet.[10] In addition to their effect on bivalves, it has been reported that *H. circularisquama* exhibits lethal effects on a microzooplankton tintinnid ciliate *Favella taraikaensis* in a cell density-dependent manner.[11,12] Frequent contact of *H. circularisquama* cells with the cytoplasm around the oral plug of *F. taraikaensis* and subsequent morphological changes of *F. taraikaensis* were observed at high flagellate cell concentrations.[12] Kim D *et al*., (2000) have also found that a microzooplankton rotifer (*Brachionus plicatilis*) is similarly

susceptible to *H. circularisquama*.[13] It has been speculated that unstable toxic substances located on the cell surface of *H. circularisquama* may be responsible for its toxicity to bivalves.[10] Although no such toxic substances have been successfully isolated and identified from *H. circularisquama* yet, it has been observed that an influx of Ca^{2+} was induced in the trochophore larvae of short-necked clams (*Ruditapes philippinarum*) after exposure to *H. circularisquama*.[14] Based on these findings, a schematic toxic mechanism of *H. circularisquama* against bivalve molluscs has been proposed.[15] Some phytoplankton species produce multiple toxins, and some of such toxins exhibit haemolytic activity. For instance, palytoxin[16] and maitotoxin[17] are known to induce a Ca^{2+} influx into mammalian erythrocytes, eventually causing haemolysis. A previous study found that *H. circularisquama* cell suspension causes marked haemolysis in rabbit erythrocytes in a cell density-dependent manner.[18,19] Furthermore, a comparative study of the haemolytic activity of several strains of *H. circularisquama* isolated from different localities in Japan suggests that haemolytic activity and toxicity to shellfish are well-correlated.[20] Since the haemolytic test is a simple and small-scale semiquantitative assay, it is useful not only for searching for toxic agents of *H. circularisquama* but also for estimating its own potential toxicity.

The raphidophycean flagellate *Chattonella* spp., such as *Chattonella marina* and *Chattonella antiqua*, are the most noxious HAB species, which cause serious damage to fish farms in Japan, especially to the aquaculture of yellowtail, *Seriola quinqueradiata*.[4] In addition to Japan, HABs due to *Chattonella* spp. often associated with fish-kill incidence have been reported in Australia, Netherlands, Brazil, and other area around the world.[21-24] Although the specific toxic mechanism of *Chattonella* spp. is still controversial, it has been suggested that suffocation is the direct cause of fish death by this flagellate species.[25-28] Previous studies have demonstrated that a decrease in partial pressure of oxygen in arterial blood is the earliest physiological change observed in fish exposed to *C. marina*.[29,30] In addition, several lines of evidence have suggested that excessive mucus on the gill surface, which is probably induced by *C. marina*, may interfere with O_2 transfer, which in turn leads to asphyxia.[27,31]

Algal polysaccharides such as alginate, fucoidan, carrageenan, laminaran, and agar have recently been drawing a great attention from diverse research fields to develop as new biomaterials, health foods, or supplements. Some of these polysaccharides show antitumor,[32-34] antiviral,[35,36] anticomplementary,[37,38] anticoagulant,[39,40] antioxidant,[41,42] anti-inflammatory,[43] and immuno-modulatory activities.[44] Brown algae such as *Fucus*

vesiculosus, *Ascophyllum nodosum* and *Laminaria angustata* are often used as raw materials for the preparation of alginate, laminaran, and fucoidan. Fucoidan is basically a sulfated fucan containing sulfated fucose as a main component and sometimes containing uronic acids, galactose, and xylose as minor compositions depending on algae species.[45] Structural characterization of sulfated polysaccharide fractions prepared from *F. vesiculosus* suggested the existence of different types of sulfated polysaccharides distinguished from fucoidans.[46] Previous study demonstrated that ascophyllan could be (xylofucoglycuronan) isolated from the brown alga *A. nodosum* as a separated fraction from fucoidan.[47] Besides some structural and bioactive similarities between fucoidan and ascophyllan,[48,49] It has also demonstrated that ascophyllan showed a growth-promoting activity on MDCK cells, while fucoidan was rather toxic to this cell line.[50] During the course of comparative studies on the cellular level biological activities of sulfated polysaccharides from different seaweed species, *F. vesiculosus* fucoidan, which is the only commercially available one, was highly cytotoxic to RAW264.7 cells, whereas ascophyllan and fucoidan isolated from *A. nodosum* showed no significant cytotoxicity on RAW264.7 cells up to 1,000 μg mL^{-1}.[51]

Porphyra species are edible red algae that are cultivated and consumed mainly in East and South-East Asian countries such as Japan, Korea, and China. Sheet-like dried foodstuff prepared from *Porphyra* species is commonly known as "nori," which is traditionally used in the Japanese cuisine sushi. Owing to the recent popularity of sushi, the market of nori is now spreading in many countries. Nori is generally known to contain some biologically functional components, such as dietary fibers, taurine, polyunsaturated fatty acids, carotenoids, and mycosporine-like amino acids (porphyra-334), as well as minerals, vitamins, and relatively high amount of proteins. Hence, dietary nori is considered to be a functional food that can contribute to human health. Porphyran, one of the main constituents of *Porphyra yezoensis* and related to agarose, is a linear sulfated polysaccharide comprising D-galactose, 3,6-anhydro-L-galactose, 6-*O*-methyl-D-galactose, and L-galactose-6-sulfate.[52-54] In addition to its health benefit as a major dietary fiber in "nori," previous studies have found that porphyran has diverse physiological activities, including antitumor, immunomodulating, antioxidant, antihyperlipidemic, and hypercholesterolemic effects.[54-59]

In this study, I studied on the biologically active substances from marine algae both HABs and seaweeds in order to further evaluate their utility potential. Chapter II contained a study on

toxicity factors in HABs and chapter III focused on the biological activities of polysaccharides isolated from seaweeds.

CHAPTER II

Toxicity Factors in Harmful Algal Blooms

Intracellular haemolytic agents of *Heterocapsa circularisquama* exhibit toxic effects on *H. circularisquama* cells themselves and suppress both cell-mediated haemolytic activity and toxicity to rotifers (*Brachionus plicatilis*)

2.1.1. Introduction

It has been reported that live *H. circularisquama* cells must come into direct contact with bivalves in order for there to be lethal effects on the bivalves, which indicates the effect may be the result of certain toxins located on the cell.[15] Based on the findings, it seems likely that the haemolytic substance on the cell surface of *H. circularisquama* is a toxin responsible for the shellfish-killing mechanism. *H. circularisquama* shows a lethal effect on a rotifer (*Brachionus plicatilis*) in a cell density-dependent manner,[13] and previous study suggested that haemolytic activity was involved in the toxicity to rotifers.[13]

Regarding the compound responsible for the haemolytic activity of *H. circularisquama*, it was found that an ethanol extract prepared from the flagellate cells showed haemolytic activity, and that this activity was light-dependent.[18,19] Purification and characterisation studies suggested that one such photosensitising haemolytic agent (named H2-a) has structural similarity to pyropheophorbide a methyl ester (PME), a well-known photosensitising haemolytic agent.[60] Comparative studies on the cytotoxicity of H2-a and PME to HeLa cells suggested that H2-a induces necrotic cell death, whereas PME triggers apoptosis.[61] Although the exact reason for this difference in types of cell death induced is still unclear, it is speculated that the relatively high affinity of H2-a to the plasma membrane might result in quick membrane damage, leading to the collapse of targeted cells without induction of apoptotic intracellular signal transduction.[61] The biological significance of the presence of a photosensitising haemolytic agent like H2-a in *H. circularisquama* for the organism itself besides a possible toxic effect on surrounding organisms of other species remains to be studied. Hence, in this study, a detailed analysis on the effects of ultrasonic-ruptured *H. circularisquama* cells on the cells themselves was conducted through a combination of haemolytic assays and rotifer-exposure experiments. The results obtained suggest the presence of intracellular agents in *H. circularisquama* cells which can not only be toxic to *H. circularisquama* cells themselves, but can also suppress live cell-mediated haemolytic activity and toxicity to rotifers. Possible impacts of ruptured *H. circularisquama* cells on HAB species, including *H. circularisquama*, are discussed.

2.1.2. Materials and methods

2.1.2.1. Plankton and rotifer cultures

Heterocapsa circularisquama was isolated from Ago Bay, Japan, and was kindly provided by Dr. Matsuyama Y. (Seikai National Fisheries Institute, Japan). *Chattonella marina* and *Chattonella antiqua* were isolated from Kagoshima Bay and Shimabara Bay, Japan, respectively. *Karenia mikimotoi* was isolated from Hiroshima Bay, Japan. These species were maintained at 26°C in 100 mL flasks containing 60 mL of a modified seawater medium (SWM-3) at a salinity of 25 in a 12:12 h photoperiod which was maintained by using a cool-white fluorescent lamp (200 ± 5 mol m^{-2} s^{-1}).[62] The modified SWM-3 contained a Tris-HCl buffer system and was autoclaved for 15 min at 121°C before use. The cell numbers of the cultures were counted microscopically using a haemocytometer (Erma Inc., Tokyo, Japan). Each flagellate culture used throughout the experiment was in its late exponential growth phase unless otherwise specified. The rotifer *Brachionys plicatilis* was kindly provided by Dr. Hagiwara A. (Faculty of Fisheries, Nagasaki University, Japan) and was cultured with Nannochlorosis oculata using the same method as described previously.[63] All the cultures were prepared using sterilised instruments. A cell-free culture supernatant of *H. circularisquama* was prepared from a cell suspension in its late exponential growth phase (4–5 × 10^5 cells mL^{-1}) through centrifugation at 5000 × g for 10 min at 4°C. The ruptured cell suspension was prepared by per- forming an ultrasonic treatment on the cell suspension (4–5 × 10^5 cells mL^{-1}) in an ultrasonic apparatus for 1–3 min at 20°C. Microscopic observation confirmed that all cells were ruptured by the treatment. Cell contents (supernatant) and cell fragments (precipitate) were prepared from the ruptured *H. circularisquama* cells through centrifugation at 15,000 × g for 10 min at 4°C. The cell-free culture supernatant and ruptured cell suspension were used immediately after their preparation for the haemolytic assay and the rotifer toxicity test.

2.1.2.2. Preparation of ethanol extract

Since it has been reported that light-dependent haemolytic agents in *Heterocapsa circularisquama* can be efficiently extracted into ethanol and maintained in a stable condition,[18] an ethanol extract was prepared for further analysis. The harvested cells from 300 mL of the cultured *H. circularisquama* in its late exponential growth phase (2 × 10^8 cells L^{-1}) were resuspended in 3 ml of ethanol and vigorously agitated with sonication at room

temperature. After centrifugation at 15,000 × g for 10 min at 4°C, the supernatant was withdrawn and stored at −30°C until its use as a cell-free ethanol extract.

2.1.2.3. Gel-filtration chromatography on a column of Sephadex LH-20

The ethanol extract prepared from 2 L of the *Heterocapsa circularisquama* culture was applied to a column (1 × 10 cm) of Sephadex LH-20 (Pharmacia, Uppsala, Sweden), equilibrated with ethanol, and eluted with ethanol. Since it has been shown that the ethanol extract contains porphyrin-related compounds with the maximum absorbance at 450nm as a main ingredient,[60] the elution was monitored for absorption at 450 nm. Separated fractions were pooled and concentrated by evaporation, and the bioactivities of these samples were examined.

2.1.2.4. Measurement of haemolytic activity

Since it has been demonstrated that *H. circularisquama* causes haemolysis of rabbit erythrocytes most potently among the erythrocytes from different species tested,[18] I conducted a haemolytic assay using rabbit erythrocytes in this study as described previously.[18] Rabbit blood was obtained from Nippon Bio-Test Laboratories (Tokyo, Japan). Erythrocytes were washed three times with phosphate-buffered saline (PBS) and put into samples adjusted to a final concentration of 4% (v/v) in modified SWM-3. Triplicate 50 μL aliquots of serial two-fold dilutions of the intact cell suspension, cell-free culture, ruptured cell suspension, ethanol extract, or other samples in modified SWM-3 were added to round-bottom 96-well plates (Becton-Dickinson, Franklin Lakes, NJ, USA). To the wells containing samples, 50 μL of a 4% (v/v) suspension of erythrocytes in modified SWM-3 were added, after which the well plates were gently shaken. After incubation for 5h at 26°C either under illumination from a fluorescent lamp (200 mol m^{-2} s^{-1}) or in the dark, the plates were centrifuged at 900 × g for 10 min at 4°C. Aliquots (50 μL) of supernatant were withdrawn from the wells and transferred to flat-bottom 96-well plates (Becton-Dickinson). The amount of haemoglobin released was determined by measuring absorbance at 560nm using a microplate reader (Multiscan GO, Thermo Fisher Scientific Inc., MA, USA). Negative controls (zero haemolysis) and positive controls (100% haemolysis) were included by using erythrocytes suspended in modified SWM-3 alone and in modified SWM-3 containing 1% v/v Triton X-100, respectively.

2.1.2.5. Rotifer exposure experiment

The rotifer exposure test was conducted in 48-well plates (Becton-Dickinson) using the method described previously.[63] Ten rotifers in each well were incubated with varying concentrations of cell suspension, cell-free culture supernatant, or ruptured cell suspension in modified SWM-3 (1 mL well^{-1}) at 26°C in either light (200 mol m^{-2} s^{-1}) or dark for the indicated periods of time (0–6 h). Then, the viable individuals, which were defined as those actively swimming in each well, were counted using a stereomicroscope.

2.1.2.6. Cytotoxicity test

The cells of *Heterocapsa circularisquama* in their late exponential growth phase were used throughout the cytotoxicity experiments. To fifty microliters of SWM-3 containing varying concentrations of ethanol extract prepared from *H. circularisquama* or pheophorbide a solution (1 mg mL^{-1} in ethanol) in 96-well plates, 50 μL of the cell suspension was added. The cells were incubated (final volume of 0.1 mL well^{-1}) in varying concentrations of each test sample in SWM-3 at 26°C either in light or dark for the indicated periods. Cells with motility were considered viable cells. The number of viable cells in the control, which did not contain a sample, was taken as 100%, and the survival in test groups treated with each sample was calculated as % of that. Ethanol alone as a control did not show any cytotoxic effect on phytoplankton species used in this study up to 4% either in dark or light.

2.1.2.7. Statistical analysis

All the experiments were repeated at least three times. Data were expressed as the mean ± standard deviation, and data were analysed with a paired Student's t-test to evaluate significant differences. $P < 0.05$ was considered statistically significant.

2.1.3. Results

2.1.3.1. Haemolytic activity of H. circularisquama under various conditions

To gain clues about the roles of haemolytic substances in the toxic effects of *Heterocapsa circularisquama* and their biological significance, I carried out detailed combination experiments with haemolytic and rotifer-exposure experiments in this study. The intact cell suspension of *H. circularisquama* used in this study, which had been cultured for more than 15 years under laboratory conditions, showed a potent haemolytic effect on rabbit erythrocytes in

a cell density-dependent manner, suggesting that haemolytic activity is a relatively stable phenotype in this dinoflagellate (Fig. 1). The cell suspension showed higher haemolytic activity in the light than in the dark, whereas the activity of the cell-free culture supernatant was significantly lower than those of the cell suspensions under both lighting conditions. Interestingly, the ultrasonically ruptured cells showed haemolytic activity only in the light, and no significant activity was detected in the dark even up to the highest concentration. Since cell-free culture supernatant showed only a weak activity in slightly light-dependent manner, small amounts of intracellular light-dependent haemolytic substances may be discharged from the cells during the cultivation. These results confirmed that *H. circularisquama* has at least two haemolytic substances with differing light dependency: one shows light-independent haemolytic activity mediated by intact cells and another one shows light-dependent activity caused by the substances released from ruptured cells.

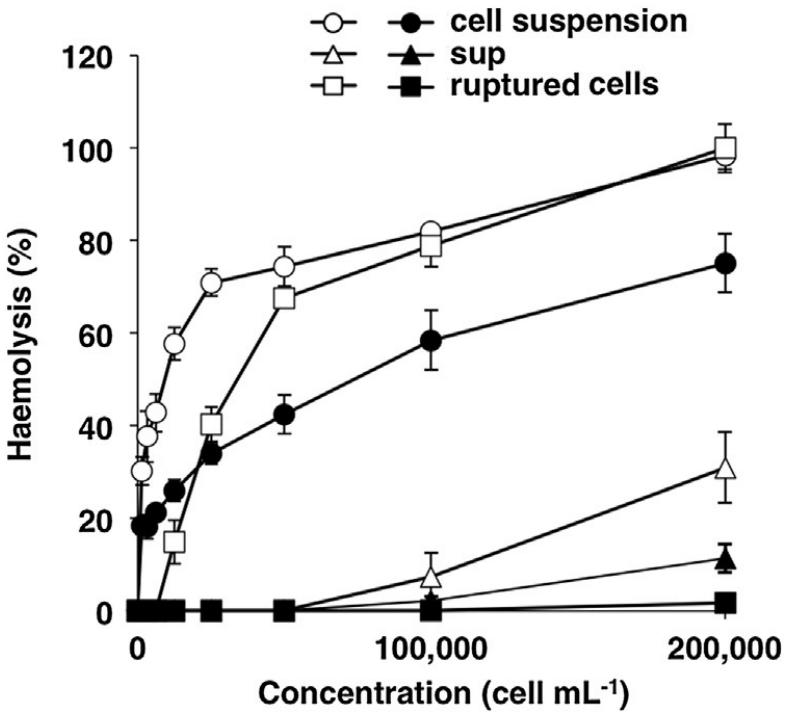

Figure 1. Haemolytic activities of live cell suspension (○, ●), cell-free cultured supernatant (△, ▲), and ultrasonically ruptured cells (□, ■) of *H. circularisquama* on rabbit erythrocytes in the light (○, △, □) and in the dark (●, ▲, ■). Each sample and all erythrocytes were incubated in 96 well-plates in a SWM-3 medium at 26℃ for 5 h, and then the haemolysis was measured as described in the text. Each point represents the average of triplicate measurements. Each bar represents standard deviation.

2.1.3.2. Toxic effect of H. circularisquama on rotifers and bioactivities of ruptured cells

The lethal effect of *H. circularisquama* on rotifers was not affected by light conditions, and almost equal toxicities to rotifers were observed both in the light and in the dark (Fig. 2). Furthermore, the ultrasonically ruptured cells showed no toxic effect on rotifers, even in the light (Fig. 3). Hence, it is obvious that intact *H. circularisquama* cells are essential to produce the toxic effect. Most likely, intact cell-mediated light-independent haemolytic activity is mainly involved in the toxicity to rotifers, whereas the light-dependent haemolytic substances detected in ruptured cells may not be related to the toxic effect of *H. circularisquama* on rotifers under the conditions tested at least when they are delivered in dissolved form. Interestingly, exogenously added ruptured cells significantly suppressed the live cell-mediated rotifer toxicity (Fig. 3), suggesting that intracellular substances discharged from ruptured cells contain some sort of suppressors against the rotifer toxicity of *H. circularisquama* itself. To search for such substances, samples of the cell contents (supernatant) and the cell fragment (precipitate) were prepared from the ruptured cells by centrifugation, and their bioactivities were examined. Most of the light-dependent haemolytic activity was observed in the cell content fraction (Fig. 4A). The cell content fraction prepared from *H. circularisquama* also showed a toxic effect on *H. circularisquama* cells themselves, and complete killing of 1×10^4 cells mL^{-1} of live *H. circularisquama* cells was attained in the presence of the cell content prepared from 12.5×10^4 cells mL^{-1} of *H. circularisquama* cells (Fig. 4B). Furthermore, in the presence of the cell content fraction, *H. circularisquama* cell-mediated light-independent haemolytic activity was significantly suppressed in a dose-dependent manner, whereas no such effect was observed in the cell fragment fraction (Fig. 4C). These results suggested that light-dependent haemolytic, cytotoxic, and cell-mediated haemolysis suppressor substances are present as soluble free form in the ruptured cell suspension, which may influence the live cell-mediated toxic behaviour, including the haemolytic activity.

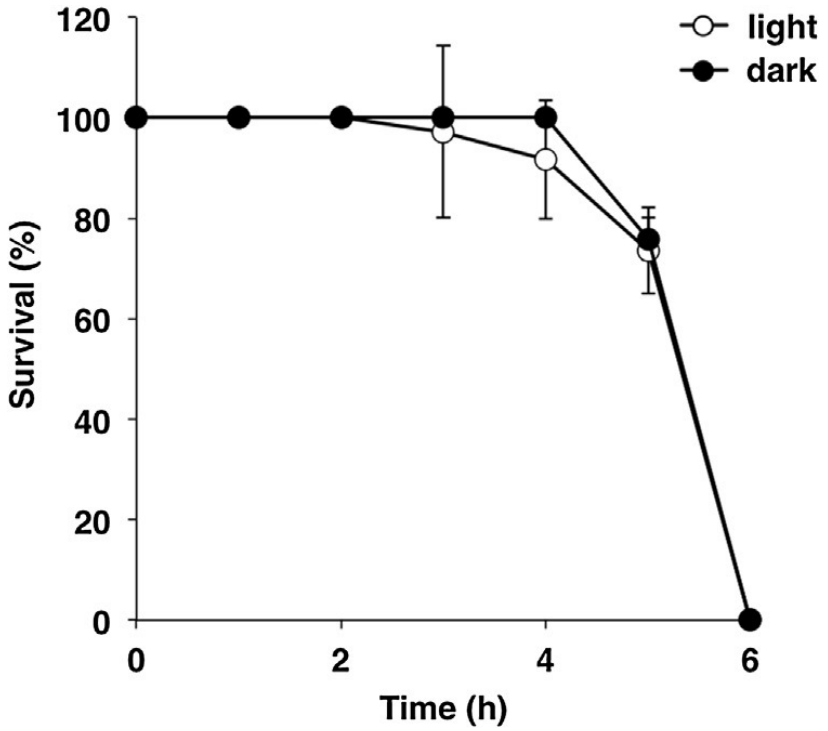

Figure 2. Toxicity of *H. circularisquama* on rotifers in the light (◯) and in the dark (●). Rotifers in 48-well plates (10 rotifers well^{-1}) were exposed to *H. circularisquama* (final 1×10^4 cells mL^{-1}) suspended in SWM-3 medium at 26℃ for the indicated periods of time, and then the number of viable rotifers remaining were counted as described in the text. Each point represents the mean of triplicate measurements. Each bar represents standard deviation.

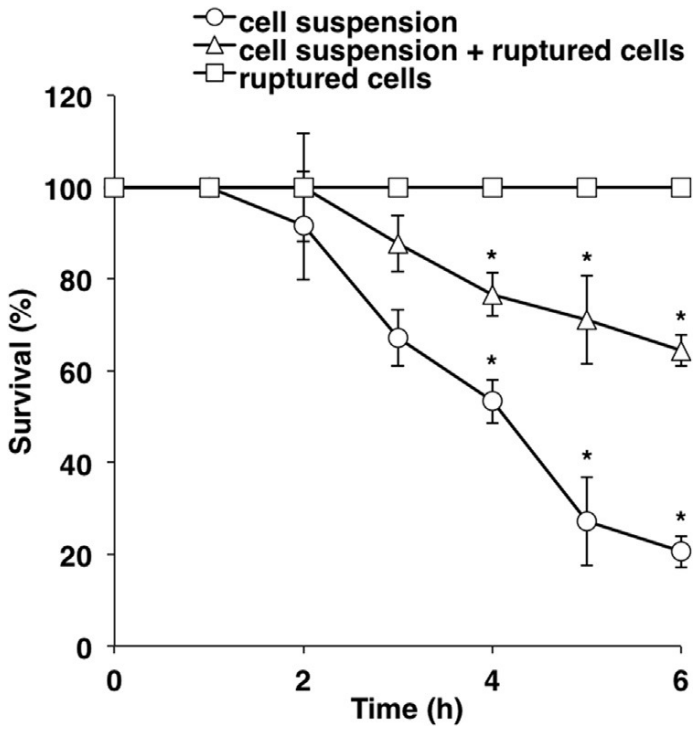

Figure 3. Effects of ultrasonically ruptured cells of *H. circularisquama* on the toxicity of *H. circularisquama* against rotifers in the light. Rotifers in 48-well plates (10 rotifers well^{-1}) were exposed to *H. circularisquama* (final 1×10^4 cells mL^{-1}) suspended in SWM-3 medium in the presence (△) or absence (◯) of ruptured cells of *H. circularisquama* (1×10^5 cells mL^{-1}) at 26℃ for the indicated periods of time, and then the numbers of viable rotifers were counted as described in the text. Toxicity of the ruptured cells of *H. circularisquama* (1×10^5 cells mL^{-1}) on rotifers (□) was examined under the same conditions. Asterisks denote significant differences between the absence and the presence of ruptured cells ($p < 0.05$).

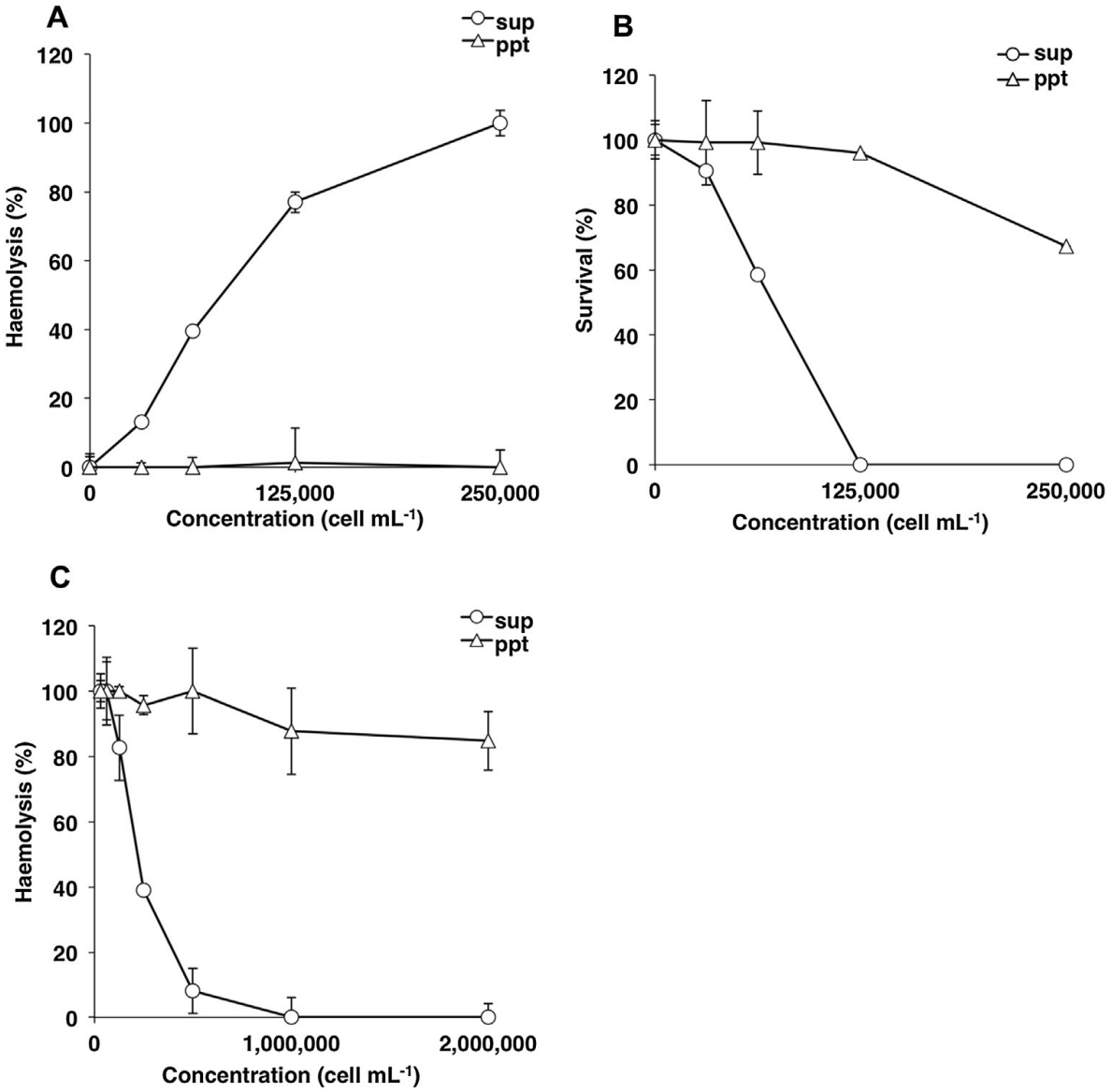

Figure 4. Bioactivities of supernatant (sup) and precipitate (ppt) fractions prepared from ultrasonically ruptured *H. circularisquama*. (A) Haemolytic activities of sup and ppt fractions in the light. Sup (◯) or ppt (△) fractions equivalent to indicated cell concentration were mixed with rabbit erythrocytes, and incubated in 96-well plates in SWM-3 medium at 26℃ for 2.5 h in the light, and then the haemolysis was measured as described in the text. Each point represents the mean of triplicate measurements. Each bar represents 1 standard deviation. (B) Toxic effects of sup and ppt fractions on *H. circularisquama* in the light. Sup (◯) or ppt (△) fraction equivalent to indicated cell concentration was mixed with *H. circularisquama* (final 1×10^4 cells mL^{-1}), and incubated in 96-well plates in SWM-3 medium at 26℃ for 24 h in the light, and then the numbers of viable *H. circularisquama* cells were

counted as described in the text. (C) Effects of sup and ppt fractions on the live *H. circularisquama* cell (final 2 × 10^5 cells mL^{-1}) -mediated haemolytic activities on rabbit erythrocytes in the dark. Sup (○) or ppt (△) fractions equivalent to indicated cell concentration were mixed with both *H. circularisquama* and rabbit erythrocytes, and then incubated in 96-well plates in SWM-3 medium at 26℃ for 5 h in the dark. The haemolysis was then measured as described in the text. Each point represents the mean of triplicate measurements. Each bar represents standard deviation.

2.1.3.3. Bioactivities of ethanol extract of H. circularisquama

Previous study found that light-dependent haemolytic activity was efficiently extracted into alcohol,[19] and that the resulting alcohol extract contains at least three haemolytic compounds with similar chemical features.[60] Among these compounds, a highly purified one named H2-a was the most potent haemolytic as well as cytotoxic agent.[60] Chemical structural analysis indicated that H2-a is a porphyrin derivative with a structure similar to pyropheophorbide a methyl ester (PME), a well-known light-dependent haemolytic agent.[60] These findings suggest that porphyrin derivatives may be responsible for the bioactivities found in the ruptured *H. circularisquama* cells. To ascertain this point, an ethanol extract was prepared from *H. circularisquama* cells, and the bioactivities of the samples were then examined. Although the ethanol extract was used without further purification in this study, which is consistent with methods of previous studies,[19,60] the ethanol extract showed potent cytotoxicity on *H. circularisquama* in a light-enhanced manner in addition to light-dependent haemolytic activity (Fig. 5A and B). In the presence of the ethanol extract, *H. circularisquama* cell-mediated haemolytic activity in the dark was also suppressed in a concentration-dependent manner (Fig. 5C). The ethanol extract induced morphological changes of *H. circularisquama* cells, leading to an increase in the round cell population during the 5 h haemolytic assay, even in the dark, though no significant decrease in total cell number of *H. circularisquama* was observed during the assay (Fig. 5D). It have demonstrated that fluorescence microscopic observation of HeLa cells treated with highly purified H2-a showed that H2-a tends to accumulate in the plasma membrane without further penetration into the cytoplasm.[61] Probably, porphyrin derivatives like the H2-a in the ethanol extract also selectively attack the cell-surface architecture of *H. circularisquama* cells, where they may inactivate haemolytic agents located on the cell surface in the dark. Upon activation on illumination, these porphyrin derivatives may cause further

severe membrane damage, leading to cell lysis.

Interestingly, the ethanol extract also showed a potent cytotoxic effect on *Chattonella marina*, *C. antiqua*, and *Karenia mikimotoi*, which are highly harmful species that cause mass mortalities of fish and shellfish, in a concentration-dependent manner at a concentration range at which no significant toxicity to rotifers is observed (Fig. 6). Although the compounds in the ethanol extract responsible for the toxicity to these harmful phytoplankton species are not identified yet, previous studies showed that the ethanol extract contained several porphyrin derivatives with photosensitising haemolytic and cytotoxic activities.[60] Hence one can speculate that the porphyrin derivatives in the ethanol extract may exhibit toxicity to phytoplankton as well. Further studies to check the susceptibility of other phytoplankton species to the porphyrin derivatives may further describe the potential impact of the porphyrin derivatives against phytoplankton, especially against harmful species.

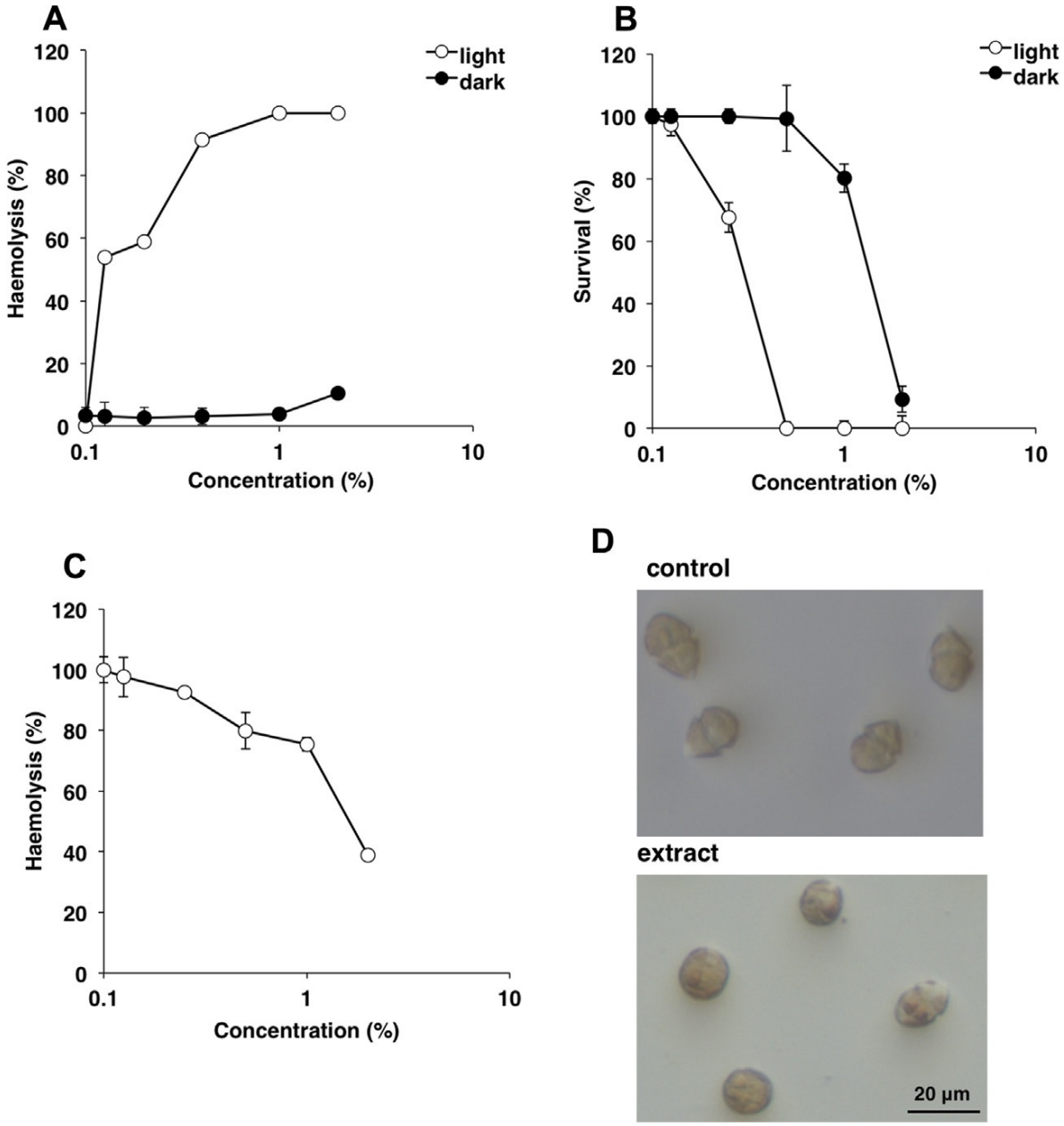

Figure 5. Bioactivities of ethanol extract of *H. circularisquama*. (A) Haemolytic activity of the ethanol extract. The ethanol extract at indicated final concentrations was mixed with rabbit erythrocytes and incubated at 26℃ for 1 h either in the light (◯) or in the dark (●), and then the haemolysis was measured as described in the text. Each point represents the mean of triplicate measurements. Each bar represents standard deviation. (B) Toxic effect of the ethanol extract on *H. circularisquama*. The indicated final concentrations of the ethanol extract were added to *H. circularisquama* (final 1×10^4 cells mL^{-1}) and incubated at 26℃ for 24 h in the light (◯) or in the dark (●). Then the numbers of viable *H. circularisquama* cells were

counted as described in the text. Each point represents the mean of triplicate measurements. Each bar represents standard deviation. (C) Effects of the ethanol extract on live *H. circularisquama* cell (final 2×10^5 cells mL^{-1}) -mediated haemolytic activities on rabbit erythrocytes in the dark. The indicated final concentrations of the ethanol extract were mixed with *H. circularisquama* and with rabbit erythrocytes and then incubated in 96-well plates in SWM-3 medium at 26℃ for 5 h in the dark. Then the haemolysis was measured as described in the text. Each point represents the mean of triplicate measurements. Each bar represents standard deviation. (D) Morphological changes of *H. circularisquama*. Micrographs of *H. circularisquama* cells after 5 h incubation without (control) or with the ethanol extract (final 2%) (extract) in the dark.

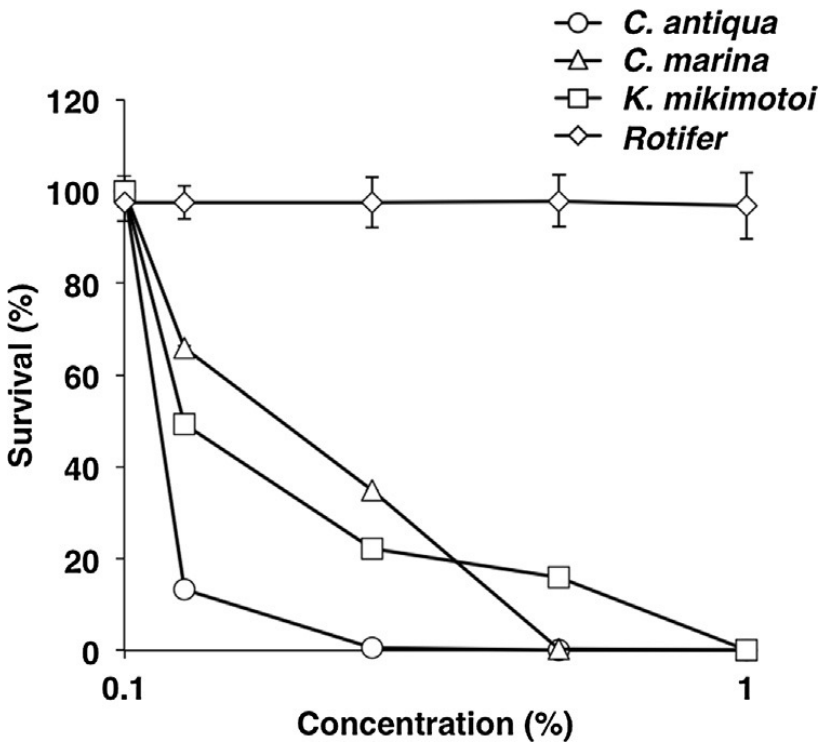

Figure 6. Toxic effect of the ethanol-extract of *H.circularisquama* on *C. antiqua* (◯), *C. marina* (△), *K. mikimotoi* (□), and the rotifer (◇). *C. antiqua* (1×10^4cells mL^{-1}), *C. marina*, (1×10^4 cells mL^{-1}), *K.mikimotoi* (1×10^4cells mL^{-1}) in 96-well plates, or rotifers (20 individual mL^{-1}) in 48-well plates were incubated with the indicated final concentrations of the ethanol extract at 26℃ for 24 h in the light, and then the viability of each cell or rotifer was determined as described in the text.

2.1.3.4. Further characterization of ethanol extract of H. circularisquama

Three fractions (f-1–f-3) were obtained from the ethanol extract in an analysis using Sephadex LH-20 (Fig. 7A). The fractions eluted later (f-2 and f-3) showed higher haemolytic activity than the fraction eluted first (f-1) (Fig. 7B), even though the optical densities measured at 450 nm of f-1, f-2, and f-3 fractions used for haemolytic assay were 24, 6, and 6, respectively. The bed volume (Vt) of the column (1 × 10 cm) of Sephadex LH-20 was 7.85 mL, and elution volumes (Ve) of f-1, f-2, and f-3 were estimated to be 14.1, 30, and 42.3 mL, respectively. Theoretically Ve/Vt values of the samples eluted under separable range should be 0.3–1.0. When the value become >1, it is suggested that interaction might occur between the gel and the samples, and elution was delayed. The Ve/Vt of f-1, f-2, and f-3 were calculated to be 1.78, 3.82, and 5.39, respectively, suggesting that elution of f-1, f-2, and f-3 did not necessarily reflect the molecular size. Hydrophobic interaction with Sephadex LH-20 may influence the elution of each fraction, and the potency of such interaction may be of the order of f-3 > f-2 > f-1. The absorption spectra of the latter two fractions were similar to that of H2-a,[61] and these fractions showed an inhibitory effect on cell-mediated haemolysis by *H. circularisquama* (Fig. 7C). These results suggest that f-2 and f-3 may contain the porphyrin derivatives responsible for cytotoxicity and the inhibition of cell-mediated haemolysis, as well as light-dependent haemolysis.

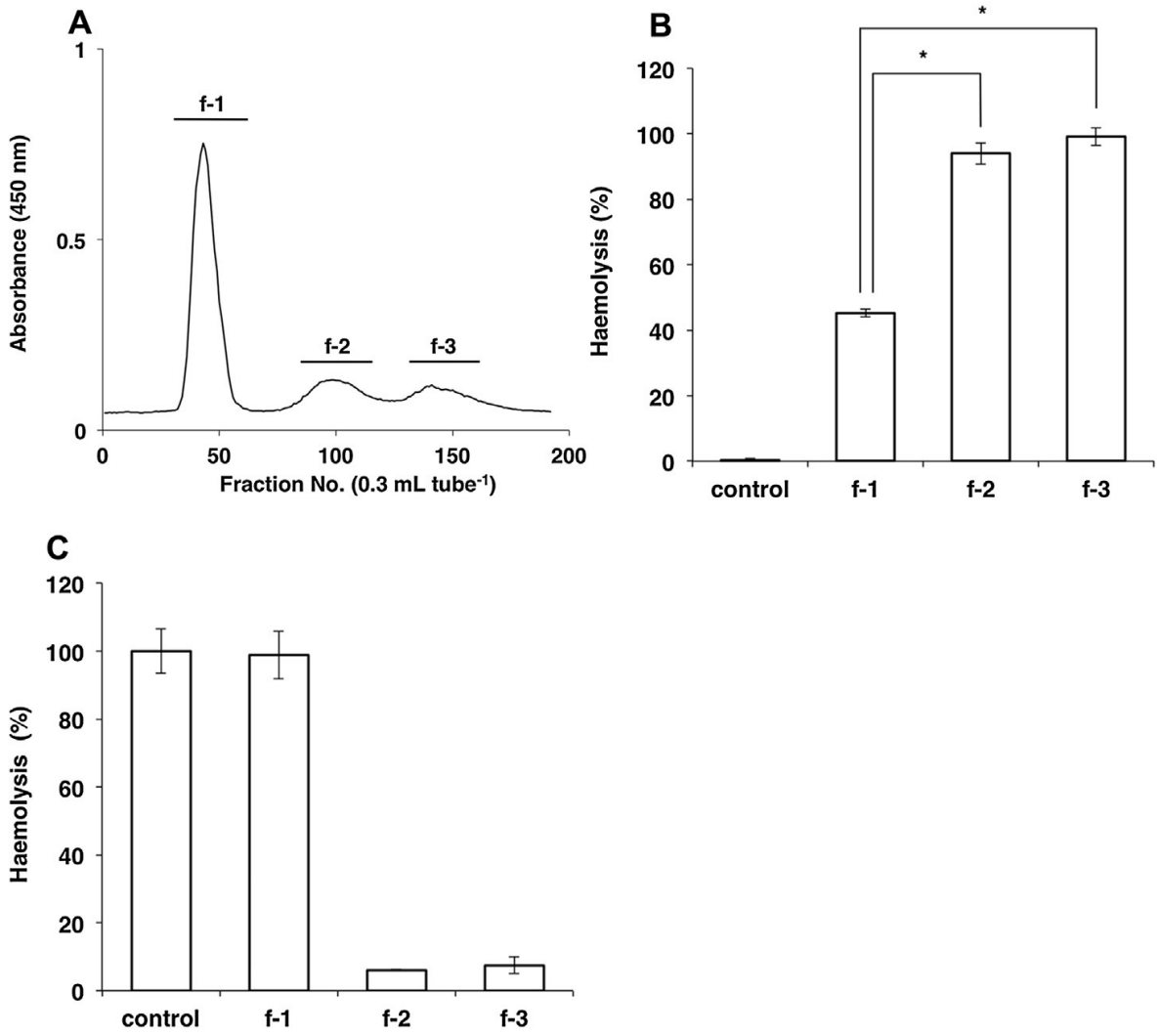

Figure 7. Analysis of the ethanol extract of *H. circularisquama* on Sephadex LH-20 and bioactivities of separated fractions. (A) Elution profile of the ethanol extract on a column of Sephadex LH-20. (B) Haemolytic activities of f-1–f-3. Each sample (final 2%) was mixed with rabbit erythrocytes and incubated at 26℃ in 96-well plates in SWM-3 medium for 1 h in the light, and then the haemolysis was measured as described in the text. (C) Effects of f-1–f-3 on the live *H. circularisquama* cell (final 2×10^5 cells mL^{-1}) -mediated haemolytic activities on rabbit erythrocytes in the dark. Each sample (final 2%) was mixed with *H. circularisquama* together with rabbit erythrocytes, and incubated in 96-well plates in SWM-3 medium at 26℃ for 5 h in the dark. Then the haemolysis was measured as described in the text. Asterisks denote significant differences between f-1 and f-2 or f-1 and f-3 ($p < 0.05$).

2.1.4. Discussion

Several porphyrin derivatives have been discovered in animal and plant sources.[64] For instance, a photosensitising porphyrin derivative was found in a methanol extract of bamboo leaves and was shown to be an inducer of apoptosis in cancer cells.[65] Pheophorbide *α* was identified as a cytokine receptor antagonist obtained from an extract of the leaves and stems of *Psychotria acuminata* (Rubiaceae), a plant used as a traditional medicine.[66] To further confirm the involvement of porphyrin derivatives in the bioactivities of ruptured cells of *H. circularisquama*, as a qualitative analysis, bioactivities of the ethanol extract were compared with ethanol solution of pheophorbide *α* (1 mg mL^{-1}). The ethanol extract at final strength of 0.4% and ethanol solution of pheophorbide *α* at final concentration of 4 g mL^{-1} caused light-dependent haemolysis with 75.5% and 57.4%, respectively. However, ethanol solution of pheophorbide *α* at final concentration of 4 g mL^{-1} exhibited no significant toxic effect on *H. circularisquama*, whereas the ethanol extract at final strength of 0.4% showed potent cytotoxicity (Fig. 5B). These results suggest that the action mechanisms of the ethanol extract may be distinct from those of pheophorbide *α*. Previous study using HeLa cells indicated that the intracellular distribution of H2-a differed significantly from that of PME, and that the LD_{50} of H2-a against HeLa cells was also more than 50 times lower than that of PME.[61] Although further studies are required to clarify the action mechanisms of *H. circularisquama*-derived porphyrin derivatives, including H2-a, previous structural analysis revealed that H2-a has two aldehyde groups that PME lacks. Aldehyde groups are generally highly reactive, and there are some reports indicating that aldehyde groups are essential for the cytotoxic activity of some toxins.[67,68] Thus, it seems likely that the characteristic action mechanism of H2-a may be partly attributed to functional side groups, such as aldehyde groups, which may explain why the peculiar bioactivities of H2-a differ from those of PME and pheophorbide a, even though they have similar porphyrin backbone structures.[60]

Several methods have been developed to mitigate the deleterious effects of HABs. Direct countermeasures remove HAB species through physical (clays, ultraviolet radiation, ultrasonic or electromagnetic waves), chemical (hydrogen peroxide, copper sulfate, surfactants), or biological control (bacteria, viruses, plankton grazers) approaches.[3,70,71,72] Application of some of these methods can be accompanied by lysis of HAB species, which may result in the discharge of intracellular substances, as was observed in this study. These results suggest that discharged substances can influence the surrounding organisms, including target HAB species.

Although the biological significances of the discharged intracellular substances are uncertain, *H. circularisquama*-derived porphyrin derivatives may facilitate the mitigation effects. A previous study showed that raphidophycean flagellates *Chattonella marina*, *Heterosigma akashiwo*, *Olisthodiscus luteus*, and *Fibrocapsa japonica* also have light-dependent haemolytic agents, which are highly toxic to HAB species.[73] It has been reported that various strains of *Alexandrium tamarense* produce diverse haemolytic substances which might be responsible for their allelopathic actions.[74] These findings suggest that the presence of haemolytic substances with various bioactivities is not limited to *H. circularisquama*. Further studies are needed to clarify the biological roles of porphyrin derivatives of *H. circularisquama*, especially in terms of the impact on plankton microenvironments.

Comparative studies on the fish-killing activities of *Chattonella marina* isolated in 1985 and *Chattonella antiqua* isolated in 2010, and their possible toxic factors

2.2.1. Introduction

In regard to the causative factor responsible for the fish-killing mechanism, it has been shown that *Chattonella* spp. generate reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (-OH).[75-79] Since ROS are toxic to living organisms,[80,81] the ROS generated by *Chattonella* spp. may be involved in gill tissue injury. This hypothesis may be supported by our previous finding that one strain of *C. marina*, which produces very low levels of O_2^-, showed lower toxicity to yellowtail than that by the other strain with higher O_2^- producing ability.[82] Since another raphidophycean flagellate *Heterosigma akashiwo* also showed ROS-mediated toxicity against rainbow trout, it seems likely that the production of ROS is a common characteristic of raphidophycean flagellates.[83]

In addition to ROS, previous studies have shown that *Chattonella* spp. produce several toxic substances, such as neurotoxins similar to the brevetoxins produced by *Karenia brevis* (formerly known as *Gymnodinium breve* and *Ptychodiscus brevis*), and hemagglutinating agents.[84-86] Matsusato and Kobayashi reported that neither the dead cells of *C. antiqua* nor cell-free filtrate of the culture of the flagellate cells was toxic to red sea bream.[87] Ishimatsu *et al*. also reported that ruptured *C. marina* showed no toxic effect on yellowtail, while there was a clear correlation between the cell's ability to produce O_2^- and its fish-killing activity.[82] These findings suggest that live cells are essential for the toxicity of *Chattonella* spp. and that the toxic factors may be quite unstable in nature. However, the exact fish-killing mechanism of *Chattonella* is still open question, and plausible candidates for the toxic factors other than ROS have not been discovered yet. It is assumed that the toxic mechanism may not be straightforward, and multiple factors are involved in the complicated processes leading to fish death caused by *Chattonella*. Recent studies have suggested that ROS and certain lipids are synergistically involved in the ichthyotoxicity through chain reaction of lipid peroxidation.[88,89]

In 2010, *C. antiqua* was isolated from Shimabara Bay, Nagasaki, Japan, where the HAB caused by *C. antiqua* resulted in serious damage to local aquacultural industries. To gain insight into the fish-killing mechanism of *Chattonella* spp., in this study, comparative experiments were conducted to determine the fish-killing activities of the above-mentioned *Chattonella* spp., using three species of fish. The effects of the newly isolated *C. antiqua* on

fish were compared to those of *C. marina* strain that was originally isolated in 1985 in Kagoshima, Japan, and has since been maintained in the laboratory, under artificial conditions.

2.2.2. Materials and methods

2.2.2.1. Cultures of Chattonella species.

The raphidophytes *C. marina* and *C. antiqua* were isolated in Japan from Kagoshima in 1985, and Shimabara Bay in 2010, respectively. These flagellate cells were maintained in 100 mL flasks containing 40-50 mL of sterilized Erd-Schreiber-modified (ESM) medium under a 12:12 h photoperiod using a cool-white fluorescent lamp (200 ± 5 μmol m^{-2} s^{-1}) at 26°C.[88] The ESM medium was autoclaved for 15 min at 121°C before use. Cell numbers were counted microscopically using a hemocytometer (Erma Inc., Tokyo, Japan). Cells from each culture were used from late exponential growth phase throughout the experiments, unless otherwise specified. Mass cultures of the flagellate cells were conducted with 5 L flasks containing 500 mL of ESM medium. To the flask, 40-50 mL of subcultured flagellate cells from late exponential growth phase was inoculated and then cultured for 10-15 days until it reached late exponential growth phase, under the conditions described above.

2.2.2.2. Preparation of ruptured C. antiqua

The ruptured cell suspension of *C. antiqua* at 10,000 cells mL^{-1} was prepared by ultrasonic treatment of the cell suspension in a bath-type sonicator (XL2020; Wakenyaku Co., Ltd., Kyoto, Japan) for 60 s at 20°C. Microscopic observation confirmed that all cells were ruptured by this treatment. The ruptured cell suspension was immediately used for the red sea bream exposure experiment and chemiluminescence analysis as described below.

2.2.2.3. Determination of chlorophyll levels

The chlorophyll content per cell was measured spectrophotometrically following acetone extraction. Each culture was harvested by centrifugation (1,000 g, 10 min) and then 100% acetone was added to the cell pellet. After vigorous agitation by vortexing, the supernatant was obtained by centrifugation (10,000 g, 10 min). The chlorophyll content of the supernatant was spectrophotometrically determined at 664 and 630 nm.

2.2.2.4. Determination of protein content

The protein content of the flagellate cells was measured with the RC-DC Protein assay kit (BIO-RAD, Hercules, CA, USA) according to the manufacturer's instruction. Bovine serum albumin (BSA) was used as a standard.

2.2.2.5. Exposure experiments

Juvenile Japanese horse mackerel (Trachurus japonicus, n = 35~50, mean body length = 10.1 ± 0.7 cm, body weight = 8.6 ± 1.8 g) and red sea bream (Pagrus major, n=50~60, mean body length = 3.9 ± 0.3 cm, body weight = 0.9 ± 0.1 g) were supplied by Nagasaki Prefectural Institute of Fisheries, Japan, in spring from April to June. Adult blue damselfish (Chrysiptera cyanea, n = 40~50, mean body length = 5.9 ± 0.3 cm, and body weight = 3.8 ± 0.5 g) were purchased from a local fish retailor in Nagasaki, Japan. This fish species was available throughout a year. Red sea breams were exposed to either ruptured or live *Chattonella* cells, in a 10 L acrylic tank. To the acrylic tank containing 2.5 L of ESM medium and 10 red sea breams, either 2.5 L of *C. marina* or *C. antiqua* cell suspension in late exponential growth phase (20,000 cells mL^{-1}) was added. As a control, 10 red sea breams were kept in 5.0 L of ESM medium. The salinity and pH of each exposure medium was 25‰ and 8.0, respectively. The experiments were carried out at 26 ± 1°C and dissolved oxygen (DO) was kept at 6.1 ± 0.6 mg L^{-1} by aeration during the exposure experiments. For Japanese horse mackerel (n = 10) and blue damselfish (n = 5), the exposure experiments were conducted in 15 and 10 L acrylic tanks with a total volume of 10.0 and 5.0 L, respectively, under the same conditions described above. To investigate the possible involvement of hydroxyl radicals in the fish-killing activity of *C. antiqua*, a blue damselfish exposure experiment was conducted in the absence or presence of sodium benzoate (final 10 and 50 mM), a hydroxyl radical scavenger.

2.2.2.6. Measurement of the superoxide anion (O_2^-)

To measure the superoxide levels of *C. marina* and *C. antiqua*, chemiluminescence analysis using L-012 (Wako Pure Chemical Industry, Co., Ltd., Osaka, Japan) as a superoxide-specific chemiluminescence probe was undertaken, as described previously.[90] Chemiluminescence responses of *C. marina* (40,000 cells mL^{-1}) and *C. antiqua* (20,000 cells mL^{-1}) were recorded for 30 s using a Bio-Orbit Luminometer (1254–001; Bio-Orbit Oy, Turku, Finland) after the addition of L-012 to each ruptured and live cell suspensions. Reaction mixtures consisted of

145 μL of each flagellate cell suspension, 50 μL of L-012 (10 μM), and either 5 μL of superoxide dismutase (SOD: Cu, Zn-SOD) solution (final concentration of 100 U mL^{-1}) or 5 μL of ESM medium. Chemiluminescence response measured in ESM medium alone was considered as a baseline for other measurements. All chemiluminescence measurements were conducted in triplicate at 26°C using 1.5 mL microtube cuvettes.

2.2.2.7. Measurement of hydroxyl radicals

The hydroxyl radical levels in the flagellate cell suspensions (40,000 cells mL^{-1}) were measured by the electron spin resonance (ESR)-spin trapping assay as described previously.[79] In brief, 10 μL of DMPO solution (89 mM) was added to 90 μL of flagellate cell suspension. After 1 h of incubation at 27°C, the reaction mixture was subjected to ESR (The EMX Plus, Bruker, Billerica, Massachusetts, USA). The measurement conditions of the ESR experiments were as follows: field sweep: 3327-3412 G, field modulation frequency: 100 kHz, modulation amplitude: 3 G, sweep time: 122.880s, time constant: 163.840 ms, microwave frequency: 9.458 GHz, microwave power: 4 mW.

2.2.2.8. Estimation of the cell count of C. marina and C. antiqua associated with gill tissues of blue damselfish exposed to flagellate cells

For the estimation of the chlorophyll levels in the gill tissue of blue damselfish, the whole gill tissue of the blue damselfish in the experiment was immediately removed from the fish after exposure to flagellate cells at 20,000 cells mL^{-1} for 1, 2, and 3 h and immersed into acetone. After 10 min of extraction, the solution was transferred to a tube and the supernatant was obtained by centrifugation (10,000 g, 10 min). The level of chlorophyll in the supernatant was estimated spectrophotometrically as described above. Based on the calibration curve between flagellate cell count and chlorophyll content, the number of flagellate cells associated with the gill was estimated. The chlorophyll level in the extract prepared from normal gill tissue using same procedure was essentially at a negligible level.

2.2.3. Results

2.2.3.1. Characterizations of C. marina and C. antiqua

The average cell lengths of *C. marina* and *C. antiqua* were 30-50 and 50-130 μm, and the widths were 20-30 and 30-50 μm, respectively. The average protein and chlorophyll contents of *C. marina* and *C. antiqua* were 4.3 and 8.0 ng per cell, and 40 and 50 pg per cell, respectively.

2.2.3.2. Fish-killing activities of C. marina and C. antiqua

As shown in Fig. 8, red sea bream exposed to the culture of *C. antiqua* at a cell density of 10,000 cells mL^{-1} exhibited mortality. The first fish died after 40 min of exposure to the flagellate cells, and all fish were dead within 3.5 h. Japanese horse mackerel and blue damselfish were also highly sensitive to *C. antiqua*, and 80% and 100% of these fish species died within a few hours of exposure, respectively. Compared to the red sea bream, these fish species were slightly more resistant to *C. antiqua*. On the other hand, *C. marina* showed no significant toxic effect on these three fish species, and no dead fish were observed even after 24 h from the time of initial exposure. The fish-killing activity of *C. antiqua* against red sea bream was dependent on the cell density, with *C. antiqua* at 5,000 cells mL^{-1} showing lower toxicity than at 10,000 cells mL^{-1}, even if 100% of the fish died within 7 h (Fig. 9).

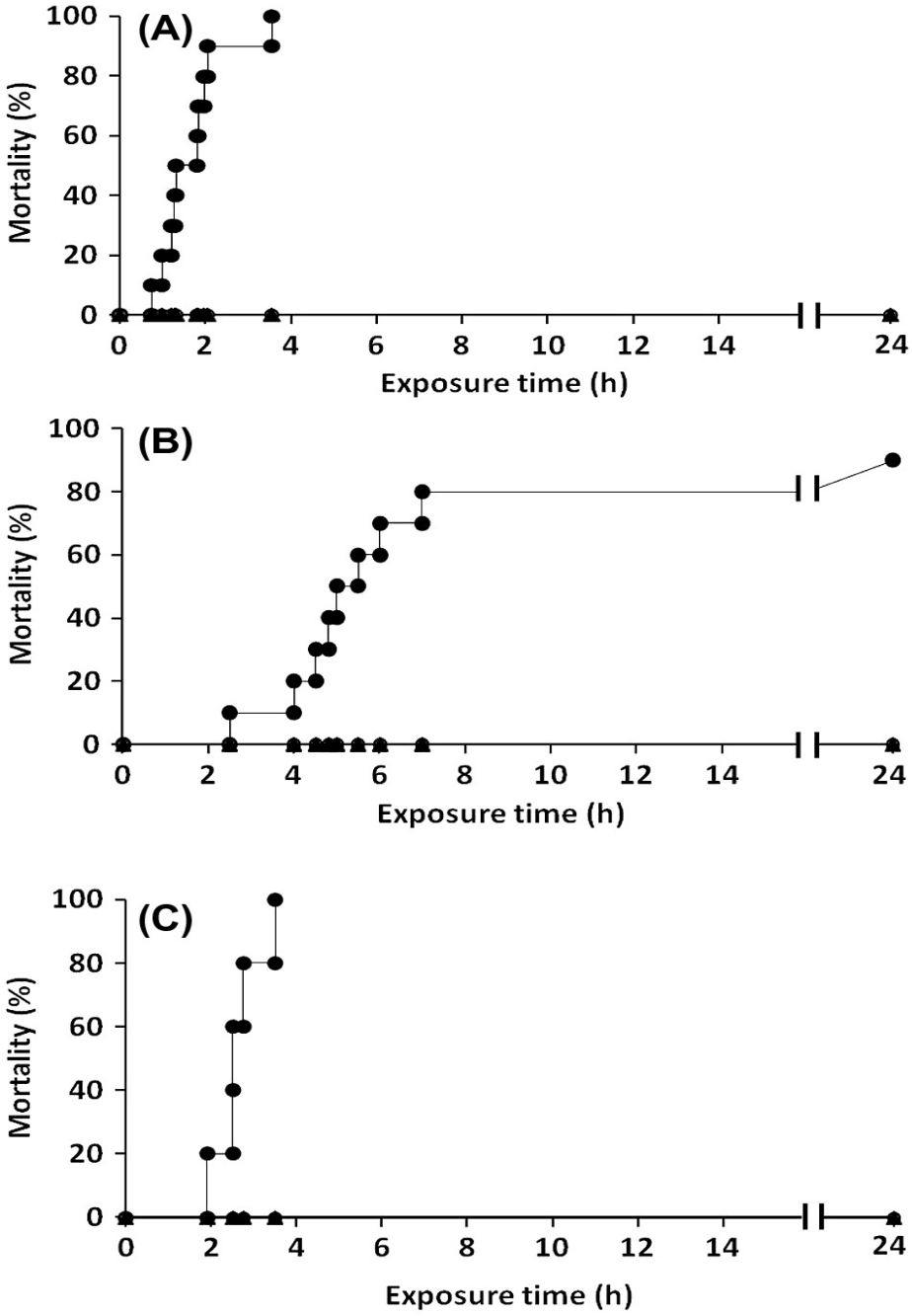

Figure 8. Fish mortality after exposure to *C. marina* and *C. antiqua*. Notes: Red sea bream (n = 10) (A), Japanese horse mackerel (n = 10) (B), or blue damselfish (n = 5) (C) were exposed to *C. marina* at 10,000 cells mL^{-1} (▲), *C. antiqua* at 10,000 cells mL^{-1} (●), or ESM medium alone (○). The mortality of each fish species was observed until 24 h after the initial exposure.

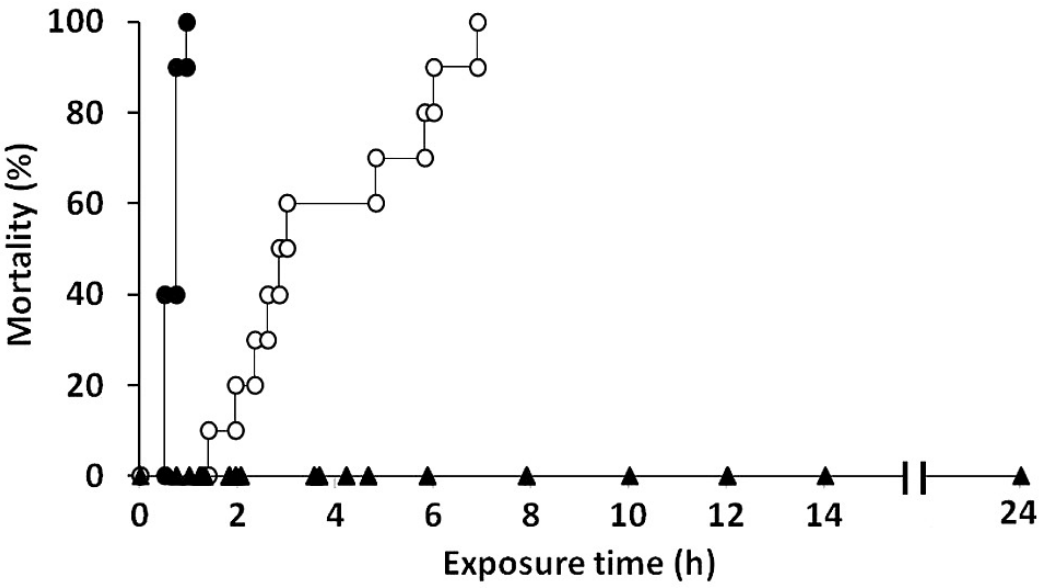

Figure 9. Cell density-dependent toxicities of *C. antiqua* against red sea bream in comparison with *C. marina*. Notes: Red sea breams (n=10) were exposed to *C. marina* at 10,000 cells mL^{-1} (▲), *C. antiqua* at 5,000 cells mL^{-1} (○) or at 10,000 cells mL^{-1} (●). The fish mortality of each group was observed until 24 h after the initial exposure.

2.2.3.3. Superoxide generating activities of C. marina and C. antiqua

Superoxide generating activities of *C. marina* and *C. antiqua* were measured by analysis of chemiluminescence. After the addition of L-012 to each cell suspension, chemiluminescence response was recorded during 30 s. As shown in Fig. 10, a strong chemiluminescence response was observed in *C. antiqua* at 20,000 cells mL^{-1}, and the response almost completely disappeared in the presence of SOD. A chemiluminescence response was also observed in *C. marina* (40,000 cells mL^{-1}); however, it was at a much lower level than that observed for *C. antiqua*, even when measured at a two-fold higher cell density.

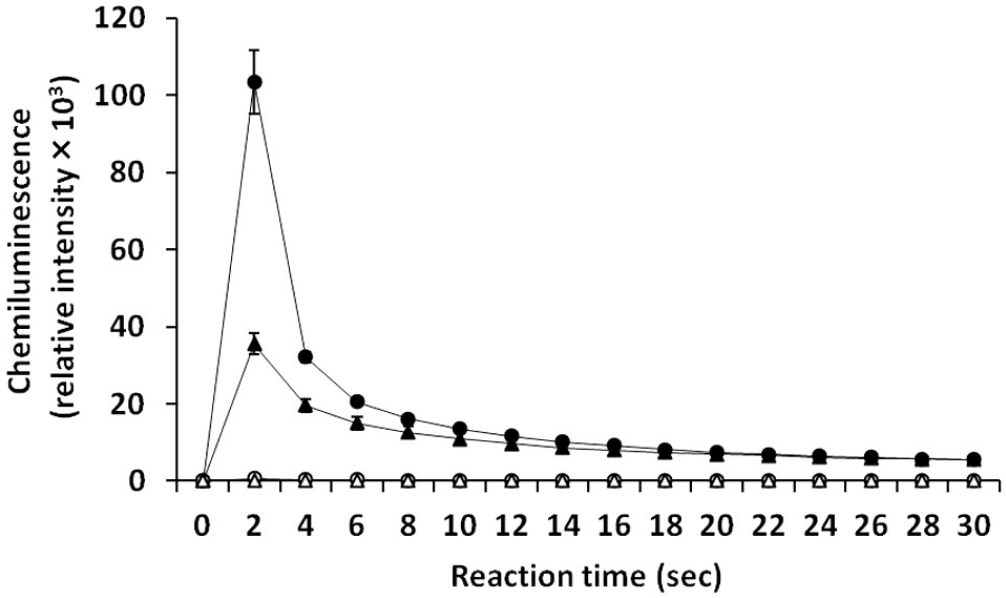

Figure 10. L-012-mediated chemiluminescence responses of *C. marina* (△, ▲) and *C. antiqua*. (○, ●). Notes: Cell suspensions of *C. marina* (40,000 cells mL^{-1}) or *C. antiqua* (20,000 cells mL^{-1}) were subjected to L-012-mediated chemiluminescence analysis in the presence (○, △) or absence (●, ▲) of superoxide dismutase (SOD) (100 U mL^{-1}). The responses were recorded for 30 s. Error bars represent average mean ± standard deviation of triplicate experiments.

2.2.3.4. Hydroxyl radical levels of C. marina and C. antiqua

As shown in Fig. 11, a noticeable signal of DMPO-OH, which reflects the presence of hydroxyl radicals, was observed in the *C. antiqua* cell suspension (40,000 cells mL^{-1}) and was much stronger than that of *C. marina* (40,000 cells mL^{-1}).

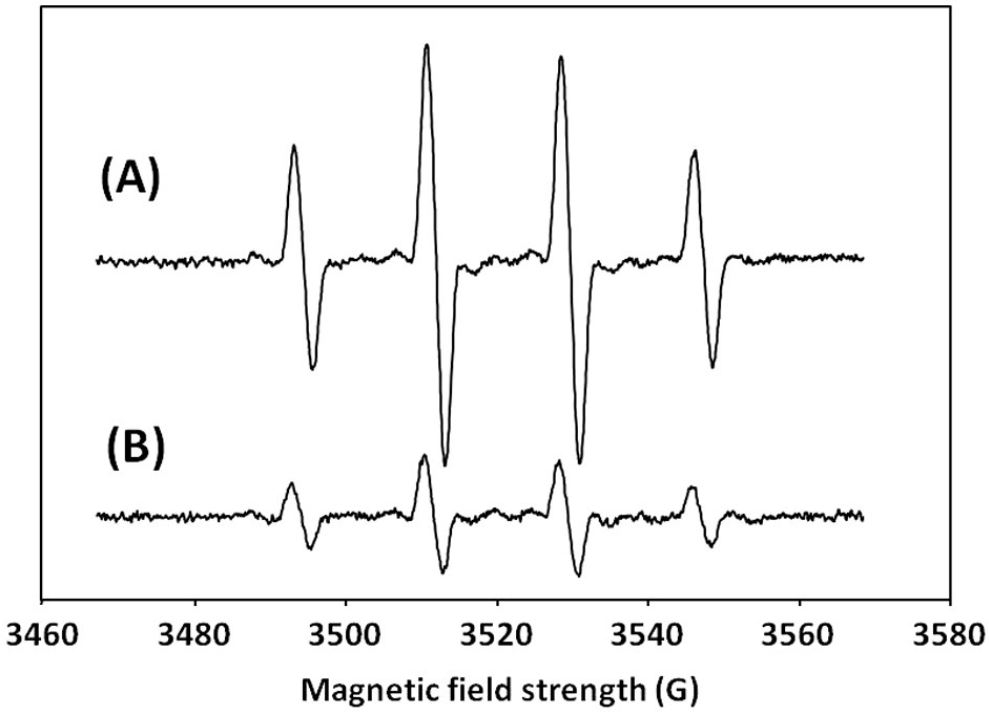

Figure 11. ESR spectra of DMPO spin adducts obtained with (A) *C. antiqua* (40,000 cells mL^{-1}) and (B) *C. marina* (40,000 cells mL^{-1}). Notes: The spectra were measured at 1 h after addition of DMPO to each flagellate cell suspension.

2.2.3.5. Effects of sodium benzoate on the fish-killing activity of C. antiqua

As shown in Fig. 12, sodium benzoate at 10 mM demonstrated partial protection against the fish-kill induced by *C. antiqua* at 10,000 cells mL^{-1}, and complete protection at 50 mM, at which concentration no dead fish were observed even after 24 h of the initial exposure.

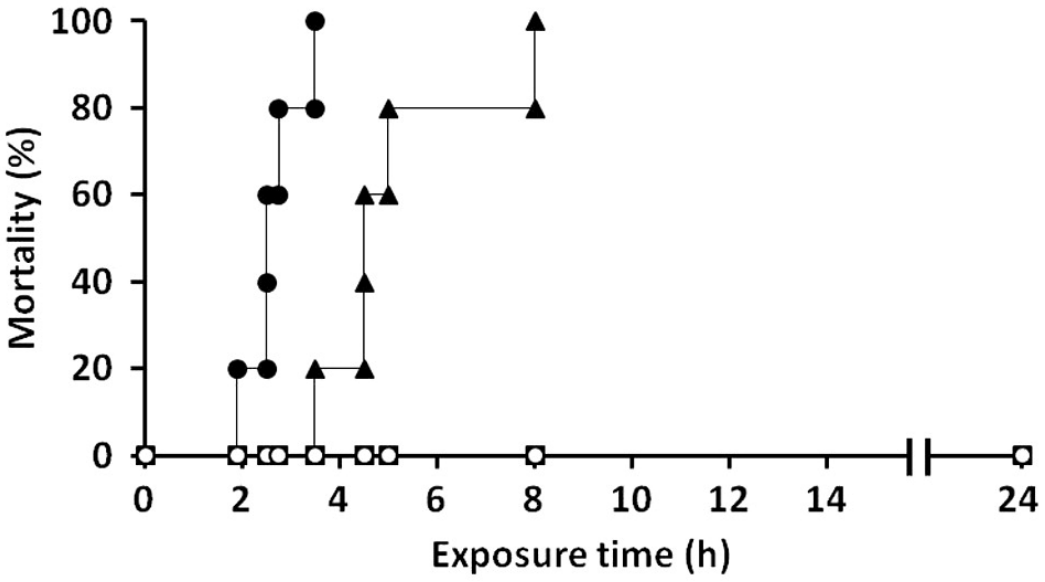

Figure 12. Effects of sodium benzoate on the mortality of blue damselfish exposed to *C. antiqua*. Notes: Blue damselfish (n = 5) were exposed to *C. antiqua* at 10,000 cells mL^{-1} in the absence (●) or presence of 10 mM (▲), 50 mM (○) of sodium benzoate, or ESM medium alone (■). The fish mortality of each group was observed until 24 h after initial exposure.

2.2.3.6. Fish-killing activity and superoxide production in ruptured C. antiqua cells

As shown in Fig. 13, ruptured *C. antiqua* cells did not demonstrate significant toxicity against red sea bream, and no dead fish were observed after 24 h of the initial exposure to ruptured cells prepared from *C. antiqua* (10,000 cells mL^{-1}). No significant chemiluminescence response was observed in the ruptured cell suspension.

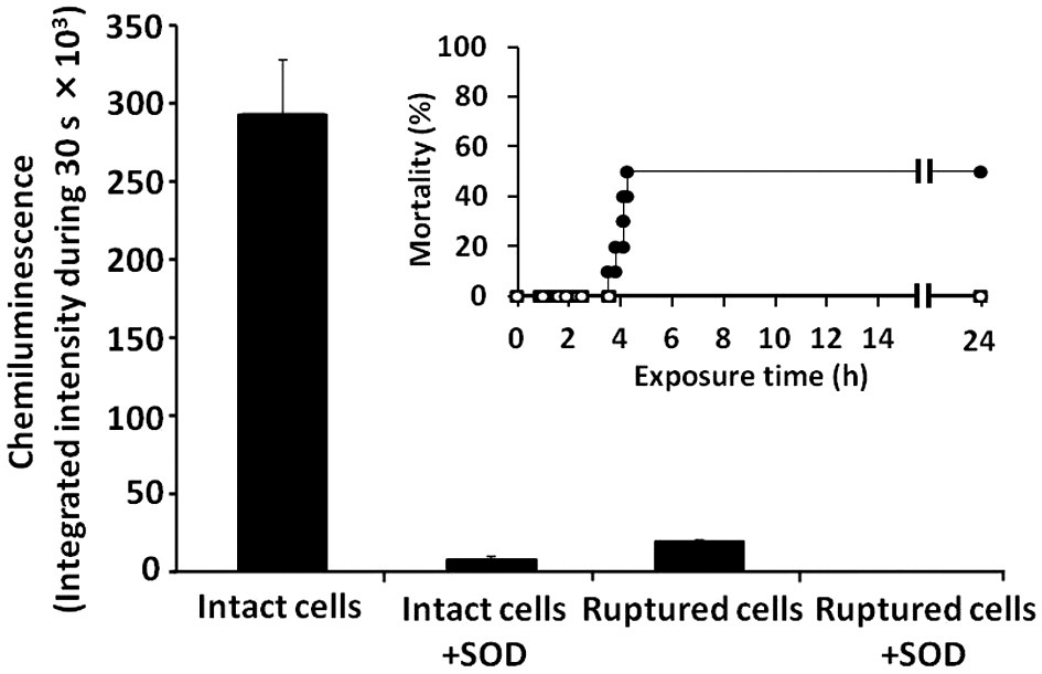

Figure 13. Fish-killing activity and ROS-producing activity of ruptured *C. antiqua* cells.
Notes: Ruptured *C. antiqua* cells prepared from a cell suspension (10,000 cells mL^{-1}) treated by sonication was subjected to chemiluminescence analysis as described in the legend of Fig. 10. The values indicate the integrated chemiluminescence intensities during 30 s obtained in intact cell suspension, intact cell suspension + superoxide dismutase (SOD) (100 U mL^{-1}), ruptured cell suspension, and ruptured cell suspension + SOD (100 U mL^{-1}). Inset shows the mortality of red sea bream (n = 10) exposed to intact cell suspension (10,000 cells mL^{-1}) (●), ruptured cell suspension prepared from same culture (○), and ESM medium alone (■).

2.2.3.7. Estimation of flagellate cell count associated with the gills of blue damselfish exposed to C. marina and C. antiqua

As shown in Fig. 14, the estimated cell numbers of *C. antiqua* gradually increased along with the exposure time, with all the values were higher than those observed during exposure to *C. marina*.

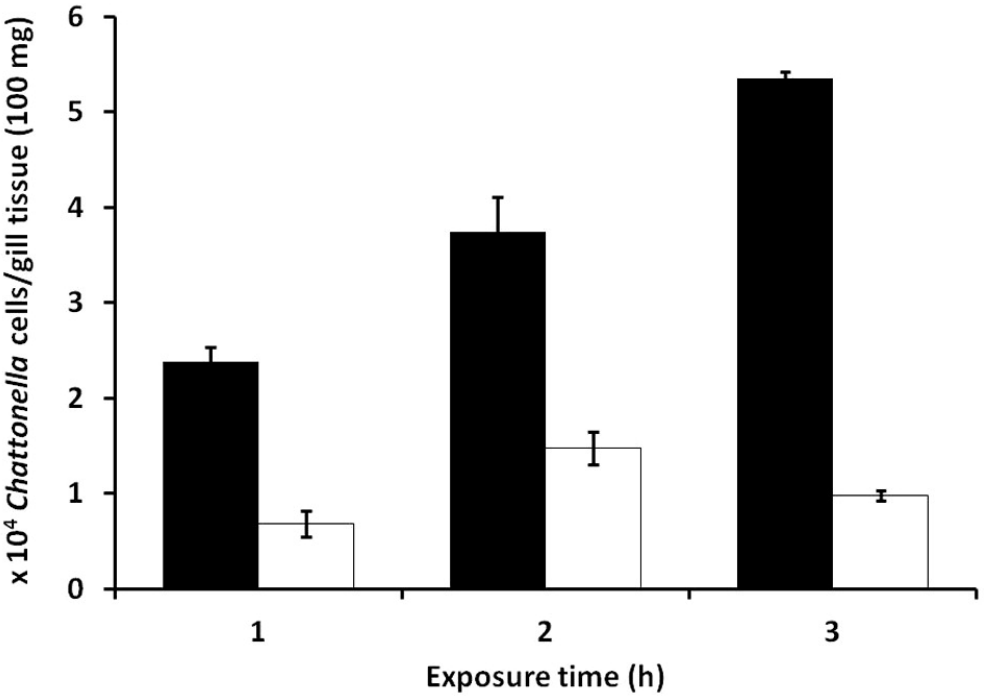

Figure 14. Estimation of cell numbers of *C. marina* and *C. antiqua* associated with the gills of blue damselfish exposed to the flagellates at 20,000 cells mL^{-1}. Notes: At 1, 2 and 3 h after exposure to *C. marina* (□) or *C. antiqua* (■), the gill of the fish was promptly excised and subjected to acetone extraction. The number of flagellate cells associated with the gill was estimated based on the calibration curve between cell number and chlorophyll level. Error bars represent average mean ± standard deviation of triplicate experiments.

2.2.4. Discussion

During the *Chattonella*-exposure experiments, the presence of *Chattonella* cells on the surface of the gill was observed. To estimate the number of *Chattonella* cells associated with the gill of the fish exposed to *Chattonella*, in this study, the amount of *Chattonella*-derived chlorophyll in the gill was extracted with acetone. The results indicated that the number of *C. antiqua* cells associated with gill tissue gradually increased with the exposure time, and the estimated cell number was greater for *C. antiqua* than for *C. marina*. These results suggest that *C. antiqua* may have a stronger affinity to gill surface in blue damselfish than that by *C. marina*. It has been reported that *Chattonella* cells have glycocalyx on their surface, and previous studies suggested that discharged glycocalyx from *Chattonella* cells was attached to the gill surface of the fish exposed to *Chattonella*, in addition to the *Chattonella* cells themselves.[91,92] Glycocalyx reportedly consists of complex carbohydrates and a carbohydrate–protein complex and was suggested to be bioactive.[91] It has been suggested that a superoxide-producing enzyme system might exist in the glycocalyx of *C. marina*.[93] Hori *et al.* reported that *C. antiqua* has lectin-like compounds and lectin receptors on the cell surface.[94] In addition, it was found that the ROS-producing activity of *C. marina* increased with the addition of fish mucus, suggesting that the interaction between fish mucus and the *Chattonella* cell surface may lead to the activation of the ROS-generation enzyme system located on the *Chattonella* cell surface.[95] The subsequent increase in ROS generation may be involved in the gill tissue damage during *Chattonella* exposure. Based on these findings, together with the results obtained in this study, it seems that the glycocalyx may play an important role in the interaction between *Chattonella* cells and gill surface, which appears to be responsible for the gill tissue damage. Further detailed analysis on the composition and structure of the glycocalyx, as well as its bioactivities, may provide an insight into the fish-killing mechanism of *Chattonella*. In this regard, our *Chattonella* strains with very different levels of fish toxicity will be useful for the elucidation of the mechanism of toxicity of *Chattonella* spp. Proteomic analysis especially focused on the glycocalyx of *C. antiqua* and *C. marina* is now planning to conduct, which may provide tips for the certain proteins linked with the differences in the fish-killing and ROS-generation activities between two strains.

Since *C. marina* has intracellular hemolytic agents even detectable in the ruptured cells, hemolytic activities of the ruptured cell suspensions of *C. marina* and *C. antiqua* were measured.[73] Although both ruptured cell suspensions showed slightly increased hemolytic

activities as compared to intact cell suspensions, no significant difference between two strains was observed (data not shown). Hence, it seems unlikely that hemolytic activity is a factor involved in fish-killing activity of *Chattonella* spp.

CHAPTER III

Biological Activities of Polysaccharides Isolated from Brown and Red Algal Species

Reevaluation of bactericidal, cytotoxic, and macrophage-stimulating activities of commercially available *Fucus vesiculosus* fucoidan

3.1.1. Introduction

Fucoidans isolated from several algal species including commercially available fucoidan have been reported to possess numerous biological activities such as anticoagulant,[39] antithrombotic activities,[40] antiviral,[96,97] antitumor, anti-inflammatory,[98,99] immuno-modulatory,[100] and apoptosis-inducing activities.[101,102] In addition to these biological activities in mammalian systems, fucoidan was found to be capable of affecting certain bacterial species. It has been reported that fucoidan extracted from *Cladosiphon okamuranus* inhibited the adhesion of *Helicobacter pylori* to human gastric cells through coating the *H. pylori* cell surface.[103] Similar activity was also observed in commercially available fucoidan from *F.vesiculosus*.[103] Several other studies reported that seaweed-derived polysaccharides showed direct antibacterial activities. For instance, crude fucoidan extracted from *Sargassum polycystum* inhibited the growth of *Vibrio harveyi*, *Staphylococcus aureus*, and *Escherichia coli* at minimal inhibition concentrations (MIC) of 12.0, 12.0 and 6.0 mg mL^{-1}, respectively.[104] Sulfated galactan with a molecular weight of 1,160 kDa extracted from marine alga *Chaetomorpha aerea* exhibited selective antibacterial activities against *Bacillus subtilis*, *Micrococcus luteus*, and *S. aureus*.[105] Polysaccharides prepared from *Laminaria japonica* showed antibacterial activities against *E. coli* and *S. aureus*.[106] These findings suggest that some sulfated polysaccharides including fucoidan have antibacterial activity, although the purity and the exact action mechanism remain to be clarified. To address how the algal source of fucoidan could associate with antibacterial activity, we first investigated antibacterial activities of commercially available *F. vesiculosus* fucoidan, along with ascophyllan and fucoidan isolated from *A. nodosum*.

Since only the *F. vesiculosus* fucoidan among these polysaccharides showed antibacterial activity, we then examined the antibacterial and cytotoxic activities after fractionation of the fucoidan with methanol-extraction, confirming such activities have derived from methanol-extractable impurities. In agreement with previous reports, methanol insoluble fraction containing fucoidan stimulated RAW264.7 cells inducing nitric oxide (NO) and tumor necrosis factor-α (TNF-α), whereas methanol extractable fraction showed no such activities. Accordingly, we recommend commercially available fucoidan should be more purified before

biochemical use to avoid misleading of its biological activities.

3.1.2. Materials and methods

3.1.2.1. Preparation of fucoidan

Fucoidan isolated from *F. vesiculosus* was purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA), and used without further purification. In this study, I mainly used fucoidan with Lot No. 066K3786 and basically the same results were obtained with fucoidan with different Lot No. 081M7672. Ascophyllan and fucoidan were prepared from powdered *A. nodosum* as described previously.[49] To distinguish two fucoidans with different origins, fucoidans from *F. vesiculosus* and *A. nodosum* were abbreviated as F-fucoidan and A-fucoidan, respectively. Polysaccharide sample solutions in phosphate buffered saline (PBS) were passed through cellulose acetate filter with pore size of 0.20 μm (ADVANTEC, Tokyo, Japan) for sterilization before use. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich, Co. Alamar blue (AB) reagent was obtained from Life Technologies, Co. (Carlsbad, CA, USA). Other chemicals were of the highest grade commercially available.

3.1.2.2. Preparation of methanol-extract and methanol-insoluble fraction of F-fucoidan

Ten mg of F-fucoidan was suspended in 1 mL of methanol, and the mixture was stirred for 24 h at room temperature. Methanol-insoluble fraction and supernatant were separated by centrifugation (2,000 × g, 5 min). The supernatant (methanol-extract) and the methanol-insoluble fraction were dried up with centrifugal vacuum concentrator (SPD131DDA; Thermo Electron Co., Milford, MA, USA). About 0.68 mg of the methanol extract as dried powder was obtained from 10 mg of F-fucoidan.

3.1.2.3. Antibacterial activity

V. alginolyticus (NBRC15630) from NITE Biological Resource Center (Chiba, Japan) and *E. coli* (JCM5491) and *S. aureus* (JCM2413) from RIKEN Biological Resource Center (Tsukuba, Japan) were used as test microorganisms. *V. alginolyticus* was cultured in marine agar or marine broth at 26°C, and *E. coli* and *S. aureus* were cultured in nutrient agar or nutrient broth at 37°C. Antibacterial activities of test samples on *V. alginolyticus* were evaluated by AB assay.[107] AB reagent is a metabolic indicator, and as consequence of bacterial growth, the

color of the AB reagent is changed from blue to pink. It has been confirmed that the classical colony formation assay and AB assay are comparable to evaluate bacterial viability.[108] The bacteria were precultured in marine broth at 26°C overnight, and harvested by centrifugation (1,500 × g). The cells were washed twice with PBS by centrifugation (1,500 × g) and the cell pellets were resuspended in PBS (final cell density of 10^7 CFU mL^{-1}), and incubated with varying concentrations of each sample at 26°C for 24 h in a 96-well flat-bottom-polystyrene microplate (100 μL $well^{-1}$) with shaking. PBS alone was used as the sample blank. After the addition of 10 μL of AB reagent to each well, the plate was incubated at 26°C for further 4 h, and then the optical density of the well at 570-600 nm was immediately recorded with a multiwell scanning spectrophotometer (Multiskan Spectrum; Thermo Electron Co., Vantaa, Finland). The blank value was subtracted from all the values as a background. For the measurement of viable bacterial cell numbers in the reaction mixture, colony formation assay was also conducted. In the assay, aliquot of 10 μL from each reaction mixture was withdrawn and inoculated into marine agar- or nutrient agar-medium after appropriate dilution with PBS, and cultured at 26 or 37°C for 24 h for colony formation.

3.1.2.4. Cell culture

RAW264.7 (mouse macrophage) cells obtained from the American Type Culture Collection (Rockville, MD, USA) were cultured at 37°C in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU mL^{-1}), and streptomycin (100 μg mL^{-1}) in a humidified atmosphere with 5% CO_2 and 95% air. Human myeloid leukemia U937 cells obtained from RIKEN Cell Bank (Tsukuba, Japan) were cultured at 37°C in RPMI1640 medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% FBS in a humidified atmosphere of 5% CO_2 and 95% air.

3.1.2.5. Cytotoxicity

Cytotoxicity of test samples on RAW264.7 cells was measured by MTT tetrazolium cytotoxicity assay. In brief, adherent cells (3×10^4 cells wel^{-1}) in a 96-well plate were cultured with varying concentrations of each sample in DMEM for 24 h, and then incubated with MTT (final 10%) for 20 min. After aspiration of the medium, dimethylsulfoxide was added to dissolve the MTT formazan reaction product and the optical density was measured at 535 nm using a multiwell scanning spectrophotometer. Cytotoxicity of test samples on U937 cells was

measured by AB assay as described previously.[109] In brief, 2×10^4 cells per well in a 96-well plate were cultured with varying concentrations of samples in the growth medium for 24 h, and then AB reagent (final 10%) was added to each well. After 2 h incubation at 37°C, the optical density of each well at 570-600 nm was measured with a multiwell scanning spectrophotometer.

3.1.2.6. Nuclear staining

Nuclear morphological changes in U937 cells treated with test samples were examined by staining with DNA-binding fluorochrome bisbenzimide (Hoechst 33342; Dojindo Laboratories, Kumamoto, Japan) as described previously.[49] In brief, cells in 96-well plates (2×10^5 cells well^{-1}) were incubated with indicated concentrations of each sample in the growth medium at 37°C, and then Hoechst 33342 (final 10 μg mL^{-1}) was added to the treated cells. After 5 min incubation at 37°C, the cells were observed by fluorescence microscope (Axiovert 200; Carl Zeiss, Inc., Jena, Germany). Cells with apoptotic nuclei, i.e., condensed or fragmented, emitting a higher intensity of fluorescence compared with normal cells, were considered as apoptotic cells. At least 100 cells at different areas were observed for each treated group to estimate % of apoptotic cell population.

3.1.2.7. Nitrite assay for the estimation of nitric oxide (NO)

To estimate NO level in RAW264.7 cells, nitrite, a stable reaction product of NO with molecular oxygen, was measured by Griess assay as described previously.[51] In brief, adherent RAW264.7 cells in 96-well plates (3×10^4 cells well^{-1}) were incubated with varying concentrations of test samples for 24 h in the growth medium at 37°C, and then the nitrite levels in the culture medium were measured.

3.1.2.8. Enzyme-linked immunosorbent assay (ELISA)

Adherent RAW264.7 cells in 96-well plates (3×10^4 cells well^{-1}) were treated with varying concentrations of test samples in the growth medium at 37°C. After 24 h incubation, the levels of TNF-α in the culture supernatants of treated cells were measured by sandwich ELISA with two antibodies to two different epitopes on TNF-α molecule as described previously.[51] The TNF-α concentrations were estimated from a reference to a standard curve for serial three-fold dilution of murine recombinant TNF-α.

3.1.2.9. Element analysis

For the analysis of carbon, hydrogen, nitrogen, sulfur and oxygen content in the methanol-extract, we used PerkinElmer 2400 Series II CHNS/O elemental analyzer (PerkinElmer, Waltham, MA, USA) according to the manufacture's instruction. Energy-dispersive X-ray detector (EDS) equipped in scanning electron microscope (JEOL JSM-7500F; JEOL Ltd., Tokyo, Japan) was used for further elemental composition investigation of the methanol-extract.

3.1.2.10. Assay for phenolic compounds

The detection of phenolic compounds in the methanol-extract was carried out by the Folin-Denis method.[110] In brief, 3.2 mL of distilled water, 0.2 mL of each sample solution or distilled water as a solvent, 0.2 mL of Folin and Ciocalteu's phenol reagent, and 0.4 mL of saturated sodium carbonate solution were mixed. The absorbance was read at 760 nm after 30 min incubation at room temperature.

3.1.2.11. Statistical analysis

All the experiments were repeated at least three times. Data were expressed as means ± standard deviation (SD). Tested groups were compared with appropriate controls using the Student's t-test. Differences were considered significant at $p < 0.01$.

3.1.3. Results

3.1.3.1. Antibacterial activities of polysaccharide samples

Antibacterial activities of F-fucoidan, A-fucoidan, and ascophyllan were assayed by AB assay using *V. alginolyticus* as a test bacterium. As shown in Fig. 15A, F-fucoidan showed antibacterial activity in a concentration-dependent manner, whereas A-fucoidan and ascophyllan exhibited even growth-promoting effects on *V. alginolyticus* rather than the growth inhibition. Since the antibacterial assays were conducted in PBS, it is considered that A-fucoidan and ascophyllan might be assimilated with the bacteria as nutrients, and that may lead to the growth promotion.

3.1.3.2. Effects of methanol-extraction on the antibacterial activities of F-fucoidan

Since it has been reported that antibacterial activities were found in organic extracts of *F. vesiculosus*,[111] I prepared methanol extract from F-fucoidan, and examined its antibacterial activities together with the methanol-insoluble residual fraction and original F-fucoidan toward *V. alginolyticus*. As shown in Fig. 15B, the extract exhibited a potent antibacterial activity that was even greater than original F-fucoidan. The methanol-insoluble fraction showed only a trace level antibacterial activity even at 1,000 μg mL^{-1}. These results suggested that methanol-extractable contaminants in F-fucoidan are mainly responsible for the antibacterial activity of F-fucoidan.

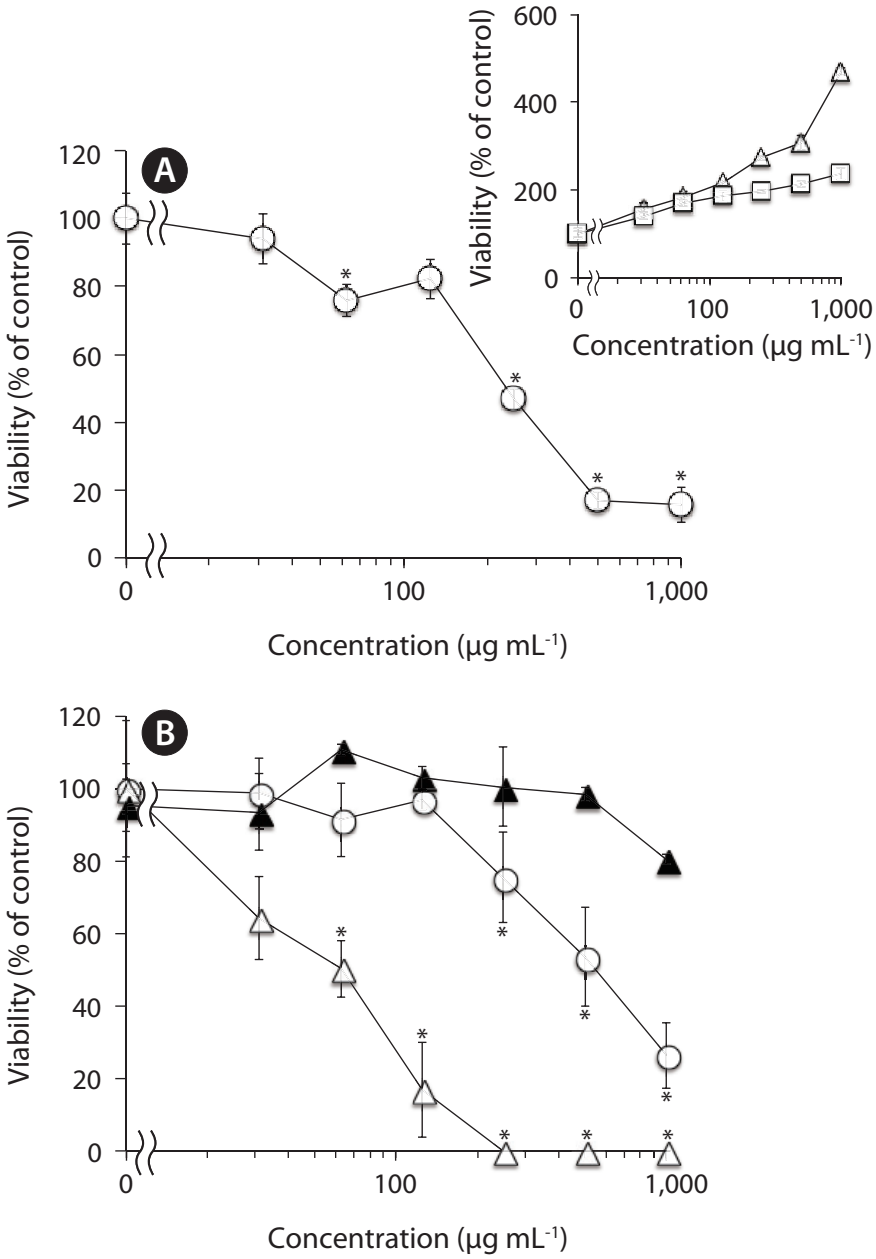

Figure 15. Effects of polysaccharides on *Vibrio alginolyticus*. (A) Effects of F-fucoidan (◯), A-fucoidan (△), and ascophyllan (□) on *V. alginolyticus* as measured by Alamar blue assay. (B) Effects of methanol-extraction on the antibacterial activity of F-fucoidan (◯), the methanol-extract (△), and the methanol-insoluble residual fraction (▲) toward *V. alginolyticus* by Alamar blue assay. Data represent the average of triplicate measurements and bars indicate the standard deviation. Asterisks indicate significant differences between with and without polysaccharide samples ($p < 0.01$).

3.1.3.3. Species-specicity of the antibacterial activities of F-fucoidan, the methanol-extract, and the methanol-insoluble residual fraction prepared from F-fucoidan

To characterize the antibacterial activities of F-fucoidan, the effects of F-fucoidan on *E. coli* and *S. aureus* in addition to *V. alginolyticus* were examined by colony formation assay. As shown in Table 1, antibacterial activity of F-fucoidan was species-specific, and the susceptibility of *V. alginolyticus* to F-fucoidan was the highest. *E. coli* and *S. aureus* showed nearly similar sensitivity to F-fucoidan. Extremely greater antibacterial activities of the methanol-extract than F-fucoidan on these bacteria were observed, whereas the activity of the methanol-insoluble residual fraction was very low.

	Unit ($\times 10^6$, CFU mL^{-1})[a]		
	V. alginolyticus	*E. coli*	*S. aureus*
PBS alone	6.2 ± 0.3	8.7 ± 0.5	5.8 ± 0.1
+ F-fucoidan	0.056 ± 0.004	2.5 ± 0.2	1.6 ± 0.09
+ Methanol-insoluble fraction	1.2 ± 0.1	7.8 ± 0.5	2.2 ± 0.03
+ Methanol-extract	0 ± 0	0 ± 0	0.057 ± 0.0001

[a]After 24 h incubation at 26°C for *V. alginolyticus* or 37°C for *E. coli* and *S. aureus* in phosphate buffered saline (PBS) in the presence of 1,000 μg mL^{-1} of each sample, the values of colony forming unit (CFU) of the treated bacteria were measured as described in the text.

Table 1. Antibacterial activities of F-fucoidan, the methanol-insoluble fraction, and the methanol-extract on *Vibrio alginolyticus*, *Escherichia coli*, and *Staphylococcus aureus* as measured by colony formation assay.

3.1.3.4. Partial characterization of the antibacterial activity of F-fucoidan

After dialysis of F-fucoidan with molecular porous membrane tube (MWCO; 6,000-8,000) against distilled water for 3 days, the contents inside the tube were lyophilized. As shown in Fig. 16, this simple dialysis of F-fucoidan could not remove the activity. Hence, the antibacterial agents in F-fucoidan may form relatively tight complex with fucoidan molecule through a hydrophobic interaction. After treatment of F-fucoidan at 121°C for 30 min, no significant reduction of the antibacterial activity was observed (Fig. 16). Furthermore, nearly 99% of antibacterial activity of the methanol-extract passed through the filter that can cut molecules with higher than 3,000 kDa (data not shown). These results suggested that antibacterial agents in F-fucoidan are low molecular weight heat-stable compounds.

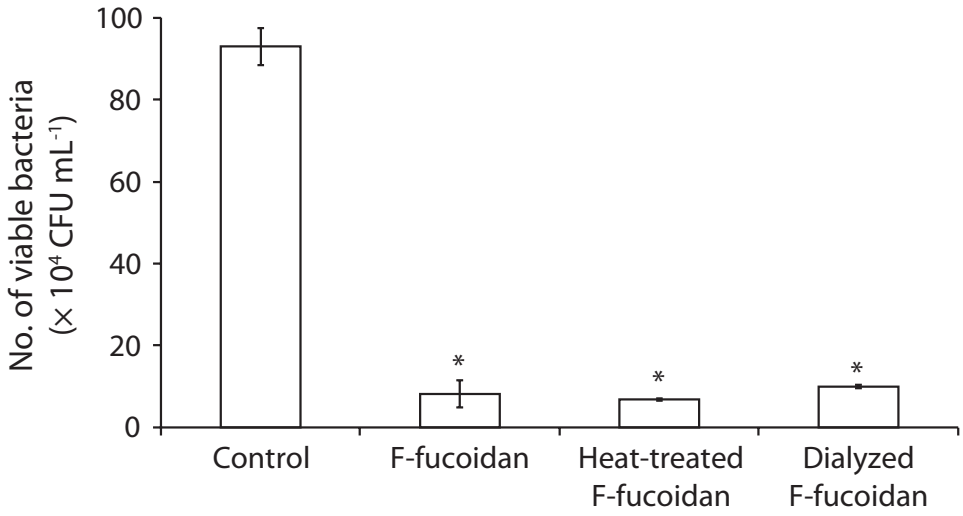

Figure 16. Effects of heat-treatment and dialysis on the antibacterial activity of F-fucoidan on *Vibrio alginolyticus* as measured by colony formation assay. The data represent the average of triplicate measurements and the bars indicate the standard deviation. Asterisks indicate significant differences between with and without test samples ($p < 0.01$).

3.1.3.5. Chemical composition analysis of the methanol-extract

The results of chemical and element analysis of the methanol-extract were summarized in Table 2. Since more than 95% of the methanol-extract were occupied with Na, Cl, K, and Si, the main ingredients of the methanol-extract are considered to be inorganic compounds such as NaCl, and organic compounds were estimated to be only about 3% (w/w). Phenolic compounds in the methanol-extract were detected by Folin-Denis method, and the amount was estimated to be less than 1% of the total weight of the extract.

Element	mg g^{-1} (%)
Cl	605.1 (60.5)[a]
Na	309.8 (31.0)[a]
K	27.9 (2.8)[a]
Si	19.4 (1.9)[a]
C	30.7 (3.1)[b]
H	3.7 (0.4)[b]
N	3.5 (0.4)[b]
Phenolic compounds	6.8 ± 1.0 (0.7)[c]

[a]Data were obtained by energy-dispersive X-ray detector analysis.
[b]Data were obtained with CHNS/O elemental analyzer.
[c]The value was estimated by the Folin-Denis method.

Table 2. Chemical composition analysis of the methanol-extract of F-fucoidan

3.1.3.6. Cytotoxic e ects of F-fucoidan, the methanol-extract, and the methanol-insoluble fraction on RAW264.7 cells and U937 cells

It have been reported that F-fucoidan, but not A-fucoidan and ascophyllan, showed cytotoxic effect on RAW264.7 cells.[51] To investigate the involvement of the methanol-extractable agents of F-fucoidan in the cytotoxicity of F-fucoidan, I examined the cytotoxic effects of the extract and the residual methanol-insoluble fraction of F-fucoidan on RAW264.7 and U937 cells. As shown in Fig. 17A, the methanol-extract showed stronger cytotoxicity on RAW264.7 cells than original F-fucoidan, and the activity of the methanol-insoluble fraction was lower than the original F-fucoidan. The extract also showed a potent cytotoxic effect on U937 cells (Fig. 17B), and nuclear staining revealed that the extract was capable of inducing typical apoptotic nuclear morphological changes in U937 cells in a concentration-dependent manner (Fig. 18A-E). Much greater population of U937 cells treated with the methanol-extract showed such apoptotic nuclear changes than the cells treated with original F-fucoidan. The activity of the methanol-insoluble fraction to induce apoptotic nuclear morphological changes was obviously lower than that of original F-fucoidan, and only a few apoptotic cells were observed in the U937 cells treated with the methanol-insoluble fraction. Time-course analysis of the nuclear morphological changes revealed that the extract induced the apoptotic changes in U937 cells with much earlier time schedule than F-fucoidan, suggesting that underlying apoptosis

inducing mechanisms of the extract and F-fucoidan might be different (Fig. 18F).

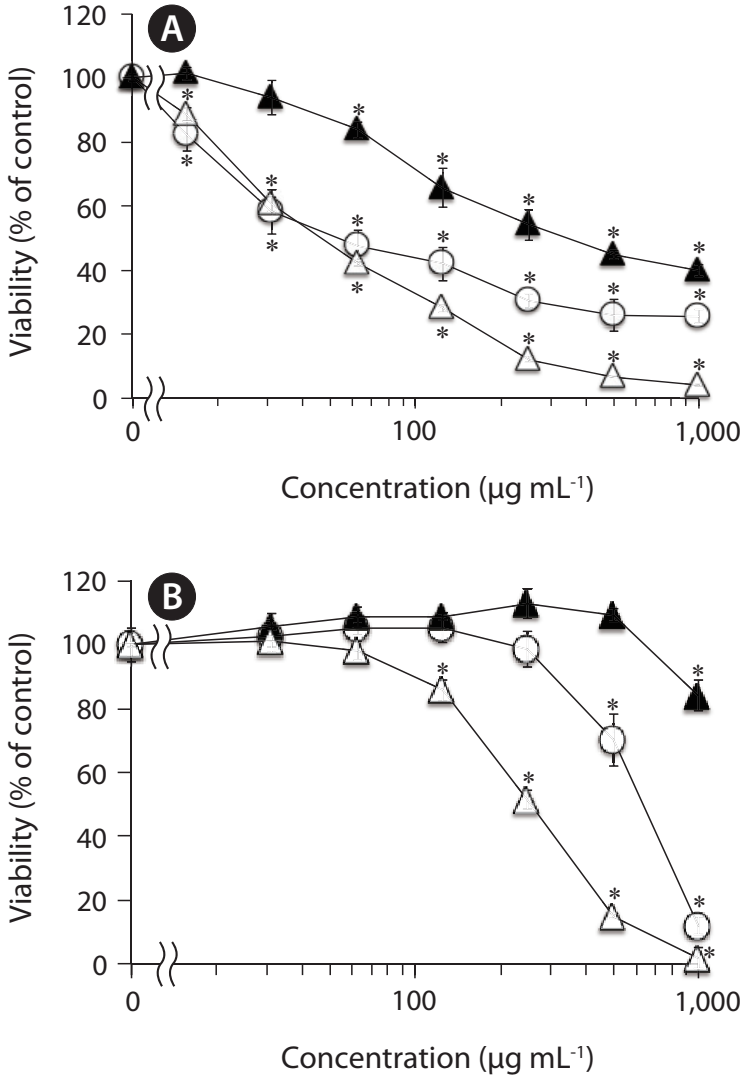

Figure 17. Cytotoxic effects of F-fucoidan, the methanol-extract, and the methanol-insoluble fraction on RAW264.7 and U937 cells. (A) MTT assay of varying concentrations of F-fucoidan (◯), the methanol-extract (△), or the methanol-insoluble fraction (▲) on RAW264.7 cells. (B) Alamar blue assay of U937 cells treated with varying concentrations of F-fucoidan (◯), the methanol-extract (△), or the methanol-insoluble fraction (▲). Data represent the average of triplicate measurements and the bars indicate standard deviations. Asterisks indicate significant differences between with and without test samples ($p < 0.01$).

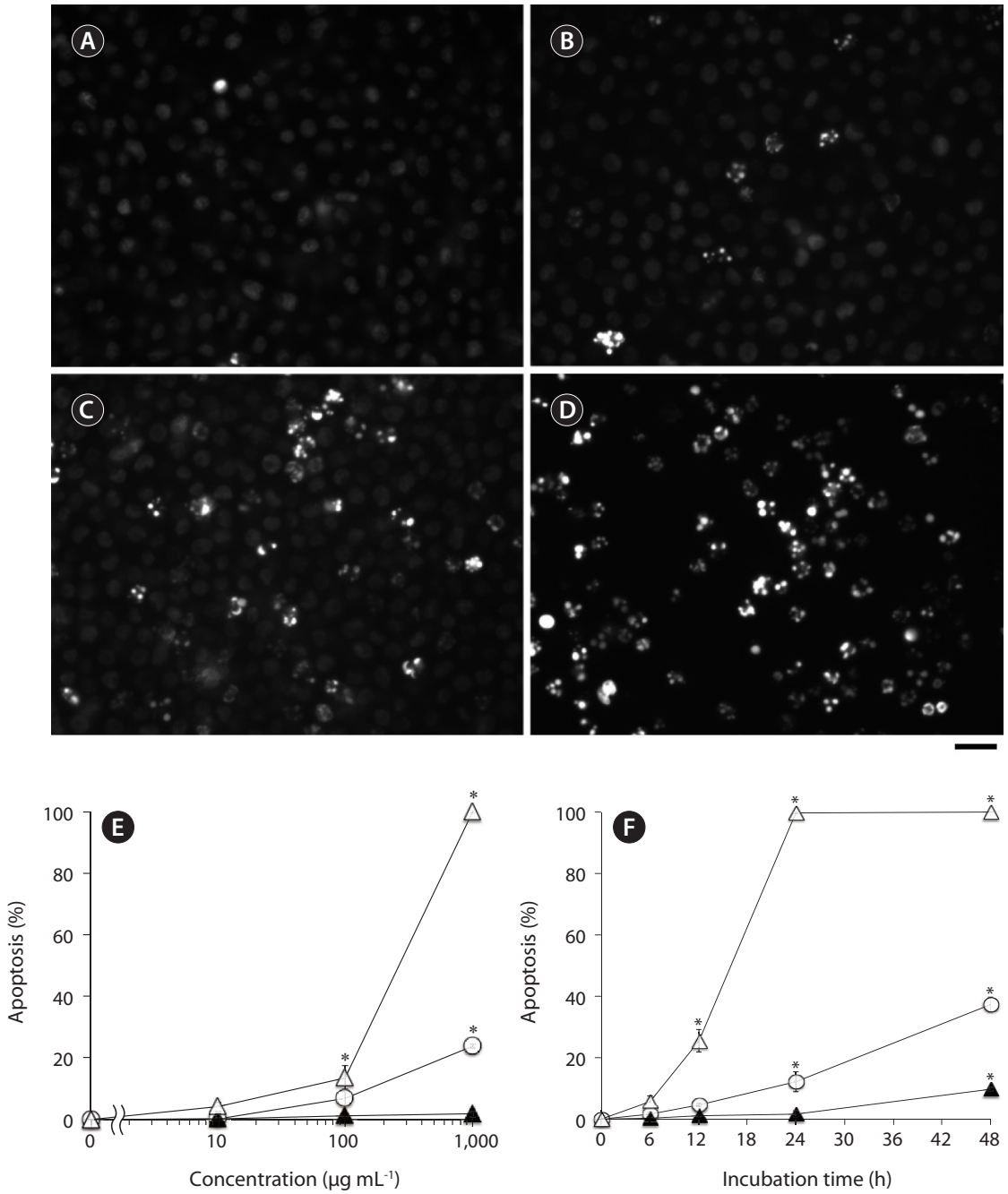

Figure 18. Nuclear morphological changes in U937 cells treated with F-fucoidan, the methanol-extract, or the methanol-insoluble fraction. (A-D) Nuclear morphological changes in U937 cells treated with medium alone (A) or with the methanol-insoluble fraction (B), F-fucoidan (C), or the methanol-extract (D) stained cells observed by a fluorescent microscope. (E) U937 cells were incubated with indicated concentrations of F-fucoidan (◯), the methanol-extract (△), or the methanol-insoluble fraction (▲) for 24 h at 37°C. The populations of the cells with apoptotic nuclear morphological changes were scored as described

in the text. (F) U937 cells were incubated with 1,000 μg mL^{-1} of F-fucoidan (◯), the methanol-extract (△), or the methanol-insoluble fraction (▲) for the indicated periods of time at 37°C and the populations of the cells with apoptotic nuclear morphological changes were scored as described in the text. Data represent the average of triplicate measurements and the bars indicate standard deviations. Asterisks indicate significant differences between with and without test samples ($p < 0.01$). Scale bar represents: A-D, 20 μm.

3.1.3.7. NO- and TNF-α-inducing activities of F-fucoidan, the methanol-extract, and the methanol-insoluble fraction in RAW264.7 cells

Fucoidans including commercially available *F. vesiculosus* fucoidan are known to induce secretion of NO and cytokines from RAW264.7 cells.[51] I investigated whether or not the methanol-extract is involved in such activities of F-fucoidan. As shown in Fig. 19, the extract showed no significant NO- and TNF-α- inducing activities, whereas the methanol-insoluble residual fraction exhibited the activities with almost equal to original F-fucoidan. These results clearly indicated that the extract does not contribute to macrophage-stimulating activity of the F-fucoidan, suggesting that the macrophage-stimulating activity of F-fucoidan is attributed to sulfated polysaccharide in F-fucoidan as a main component.

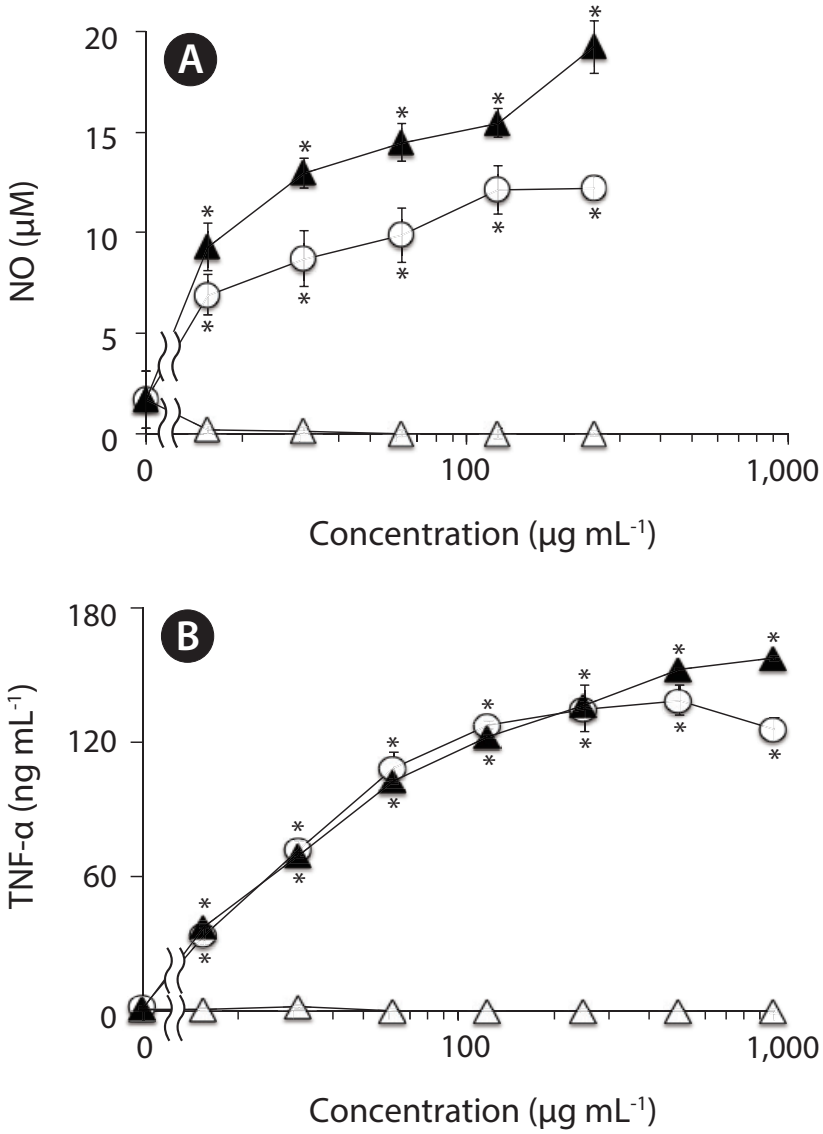

Figure 19. Nitric oxide (NO)- and tumor necrosis factor-α (TNF-α)- inducing activities of F-fucoidan, the methanol-extract, and the methanol-insoluble fraction in RAW264.7 cells. Adherent RAW264.7 cells were incubated with varying concentrations of F-fucoidan (◯), the methanol-extract (△), or the methanol-insoluble fraction (▲). (A) The NO levels in the culture medium from the treated cells were estimated by Griess assay. (B) The levels of TNF-α in the culture supernatants of the treated cells were measured by enzyme-linked immunosorbent assay as described in the text. Data represent the average of triplicate measurements and the bars indicate standard deviations. Asterisks indicate significant differences between with and without test samples ($p < 0.01$).

3.1.4. Discussion

The results indicated that only the F-fucoidan showed growth inhibitory effect on *V. alginolyticus* in a concentration-dependent manner with 500 μg mL^{-1} of MIC, and the activity was significantly reduced by methanol-extraction. Furthermore, the methanol-extract showed even stronger antibacterial activity than original F-fucoidan, suggesting that the most part of antibacterial activity of F-fucoidan is derived from non-polysaccharide contaminants. Although the bioactivities of algal polysaccharides differ depending on the compositions, the entire structures, and the physicochemical properties,[112,113] at least ascophyllan and A-fucoidan showed no significant growth-inhibitory effect on *V. alginolyticus* up to 1,000 μg mL^{-1}. Based on our results, we propose that at least the process of methanol-extraction should be conducted on commercially available *F. vesiculosus* fucoidan to avoid misleading of its biological activity.

Moreover, the methanol-extract prepared from F-fucoidan exhibit much stronger cytotoxic effects on RAW264.7 and U937 cells than original F-fucoidan, while the cytotoxicities of F-fucoidan decreased after methanol-extraction. Further study showed that the extract was capable of inducing apoptotic nuclear morphological changes in U937 cells. Time-course analysis on the appearances of apoptotic cells showed that the kinetics of the extract to induce apoptosis might differ from that of original F-fucoidan. In other words, F-fucoidan may be contaminated with apoptosis-inducing agents that have a distinct action mechanism from fucoidan itself. The cytotoxicity and apoptosis-inducing activity of F-fucoidan on U937 cells could not completely be eliminated by methanol-extraction.

Partial chemical characterization suggested that the agents responsible for the antibacterial activity in the methanol-extract were low molecular weight heat-stable compounds. Element analysis suggested the methanol-extract contains phenolic compounds in addition to inorganic compounds such as NaCl. It has been reported that phenolic compounds such as phlorotannins present in brown algae showed antibacterial activities against Gram-positive and Gram-negative bacteria with relatively broad antibacterial spectrum.[114] Similar to phlorotannins, the methanol-extract of F-fucoidan showed significant antibacterial activities on *S. aureus* and *E. coli* in addition to *V. alginolyticus*. Furthermore, apoptosis-inducing activity of phlorotannins has been reported.[115] Hence, one possible candidate for the antibacterial and cytotoxic agents in the methanol-extract are phenolic compounds, although further studies are required to identify and characterize these agents responsible for these activities.

In addition, the methanol-insoluble residual fraction of F-fucoidan exhibited the activities to induce NO and TNF-α from RAW264.7 cells with almost similar concentration-dependent profile to the original F-fucoidan, while the methanol-extract showed no significant activities over the concentration range (0-1,000 μg mL^{-1}) tested, suggesting that the macrophage-stimulating activities of F-fucoidan are mainly attributed to sulfated polysaccharide as a main component.

Protective effect of porphyran isolated from discolored nori (*Porphyra yezoensis*) on lipopolysaccharide-induced endotoxin shock in mice

3.2.1. Introduction

A previous study demonstrated that porphyran prepared from *P. yezoensis* inhibited nitric oxide (NO) production from lipopolysaccharide (LPS)-stimulated RAW264.7 mouse macrophages in a concentration-dependent manner through the inhibition of NF-κB activation.[116] These results suggest that porphyran can exhibit anti-inflammatory activity. Furthermore, recent study showed that discolored nori with no commercial value contains a much higher amount of porphyran than normal nori.[117] Chemical and biological analyses revealed that porphyrans obtained from discolored nori (dc-porphyran) have relatively lower molecular size than those from normal nori (n-porphyran), and dc-porphyran exhibits greater antioxidant activity than n-porphyran. Interestingly, dc-porphyran showed potent inhibitory effect on NO production in LPS-stimulated RAW264.7 cells by preventing the expression of inducible NO synthase.[117] Furthermore, it have been demonstrated that dc-porphyran was separated into four fractions (F1–F4) by DEAE-anion exchange chromatography, and one fraction (F1) showed the highest inhibitory effect on NO production from LPS-stimulated RAW264.7 cells. These findings suggest that dc-porphyran, especially F1, might be useful for the treatment of inflammatory diseases and endotoxin shock.[114]

To evaluate this possibility, in this study, I examined the effect of dc-porphyran and F1 on LPS-induced endotoxin shock in mice.

3.2.2. Materials and methods

3.2.2.1. Materials

Dc-porphyran was prepared from discolored nori, and F1 was separated from dc-porphyran by DEAE-chromatography as described previously.[117] LPS from Escherichia coli 0111: B4 (purified by phenol extraction) was purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). For experiments in vivo, dc-porphyran, F1, and LPS were dissolved in pyrogen-free saline. TNF-α capture antibody and anti-mouse TNF-α monoclonal antibody were purchased from Thermo Fisher Scientific Inc. (MA, USA). Other chemicals were of the highest grade commercially available.

3.2.2.2. Animal and treatment

Specific pathogen-free (SPF) male ddY mice (4 weeks old, 17.0-26.0 g) were obtained from Texam, Nagasaki, Japan. These mice were housed at a constant 24℃ under controlled conditions of 12 h light/dark cycles and provided with free access to laboratory standard food CE-2 (Texam, Nagasaki, Japan) and water. All animal experiments were conducted according to the Guidelines of the Japanese Association for Laboratory Animal Science and the Guidelines for Animal Experiments of Nagasaki University (No. G07002), Japan. Dc-porphyran or F1 (10 or 100 mg kg^{-1}) was administered intraperitoneally (i.p.) 60 min prior to LPS (30 mg kg^{-1}) i.p. injection, or intravenously (i.v.) together with LPS (20 mg kg^{-1}). Saline alone was used as control. Five mice were used for each test group (n = 5). The mortality of mice was recorded for 80 h after LPS injection in each test group. Blood samples were collected and the liver was excised after i.p. administration of an anaesthetic (a mixture of 0.75 mg kg^{-1} medetomidine hydrochloride, 4 mg kg^{-1} midazolam and 5 mg kg^{-1} butorphanol tartrate) at 12 h after LPS injection.

3.2.2.3. Measurement of serum TNF-α levels

The level of tumor necrosis factor (TNF)-α in the serum of each treated mouse was measured by sandwich enzyme-linked immunosorbent assay (ELISA) with two antibodies to two different epitopes on the mouse TNF-α molecule by a method similar to that described in a previous study.[116] The TNF-α concentrations were estimated from reference to a standard curve for a serial two-fold dilution of murine recombinant TNF-α.

3.2.2.4. Nitrite assay for the estimation of nitric oxide (NO)

The level of nitrite, a stable reaction product of NO with O_2, was determined in the serum from each treated mouse using the Griess assay method as described in a previous study.[118] In brief, each serum sample was deproteinized by addition of sulfosalicylic acid and subsequent incubation for 30 min at 24℃. After centrifugation (10,000 × g for 15 min), the supernatant (100 μL) of each sample was mixed with 5% NH_4Cl buffer (150 μL) and 5% NaOH (30 μL). To convert nitrate to nitrite, a working solution (0.31 M phosphate buffer, pH 7.5, 0.1 mM FAD, 1 mM NADPH, and 10 U mL^{-1} nitrate reductase) was added to the reaction mixture and the reaction was allowed to proceed in the dark for 1 h. The final reaction mixture was added to an equal volume of Griess reagent (3 mM sulfanilic acid and 30 M

N-1-naphthyl-ethylenediamine dihydrochloride, and 25% glacial acetic acid) in 96-well plates and incubated for 10 min at 24℃. Nitrite levels were determined by measuring the absorbance at 540 nm using a MULTISCAN GO (Thermo Fisher Scientific Inc., MA, USA). A calibration curve was made with known concentrations of $NaNO_2$ standard solution.

3.2.2.5. Measurement of lipid peroxidation

Malondialdehyde (MDA), an indicator of lipid peroxidation in liver homogenates, was determined by the thiobarbituric acid-reactive substance method.[119] The absorbance of the reaction product was detected at 532 nm, and the MDA levels were calculated as nmol g^{-1} protein.

3.2.2.6. Preparation of peritoneal cells and macrophages

At 12 h after i.v. injection of LPS (20 mg kg^{-1}) together with dc-porphyran or F1 (100 mg kg^{-1}), peritoneal cells were harvested by peritoneal lavage with 10 mL cold Dulbecco's modified Eagle's minimum essential medium (DMEM), which yielded 1-2 × 10^6 cells $mouse^{-1}$. After washing twice with DMEM by centrifugation at 200 × g, the cells were resuspended in DMEM supplemented with 10% FBS. The cells were placed in 96-well plates at a density of 8 × 10^4 cells $well^{-1}$, and were incubated for 24 h at 37℃. Then the medium was removed and NO and TNF-α levels in the medium were measured as described above. For the analysis of inducible NO synthase (iNOS) mRNA, monolayers of peritoneal macrophages were isolated from peritoneal cells as described previously.[120] In brief, 1 × 10^6 peritoneal cells harvested from mice after 12 h i.v. injection of LPS (20 mg kg^{-1}) together with dc-porphyran or F1 (100 mg kg^{-1}) were allowed to adhere to the 35 × 10 mm tissue culture dish by culturing them for 2 h at 37℃ in CO_2 incubator. Non-adherent cells were removed by gently washing three times with warm PBS. More than 90% of the adherent cells were morphologically macrophages. The adherent cells (3 × 10^5) were subjected to reverse transcription-polymerase chain reaction (RT-PCR) as described previously[116].

3.2.2.7. Statisticalanalysis

All the experiments were repeated at least three times. Data were expressed as means ± standard deviation (S.D.). Tested groups were compared with appropriate controls using Student's t-test. Differences were considered significant at $p < 0.05$.

3.2.3. Results

3.2.3.1. Protective effects of dc-porphyran and F1 on LPS-induced mortality in mice

A previous study have been found that dc-porphyran, especially F1, a fraction prepared from dc-porphyran, showed in vitro anti-inflammatory activity superior to that of porphyran prepared from normal nori.[117] Hence, in this study, I evaluated the ability of dc-porphyran and F1 to protect mice from LPS-induced mortality. When dc-porphyran or F1 (100mg kg^{-1}) was i.p. administered 1h before LPS (30 mg kg^{-1}) i.p. injection, LPS lethality was completely suppressed (Fig. 20A). At a lower dose (10 mg kg^{-1}), both porphyran samples showed a partial protective effect, and F1 was slightly more effective than dc-porphyran (Fig. 20A). Intravenously administered LPS (20 mg kg^{-1}) was considerably more lethal than i.p. administered LPS, and caused 100% mortality within 48 h. Intravenous administration of F1 (100 mg kg^{-1}) together with LPS resulted in a significant increase in survival rate to 60% after 80 h, whereas dc-porphyran enhanced the toxic effect of LPS, and all the mice died within 24 h (Fig. 20B). At a lower dose of dc-porphyran and F1 (10 mg kg^{-1}), both porphyran samples showed similar protective effect on LPS- induced lethality, with 40% survival rate 80 h after LPS injection (Fig. 20B).

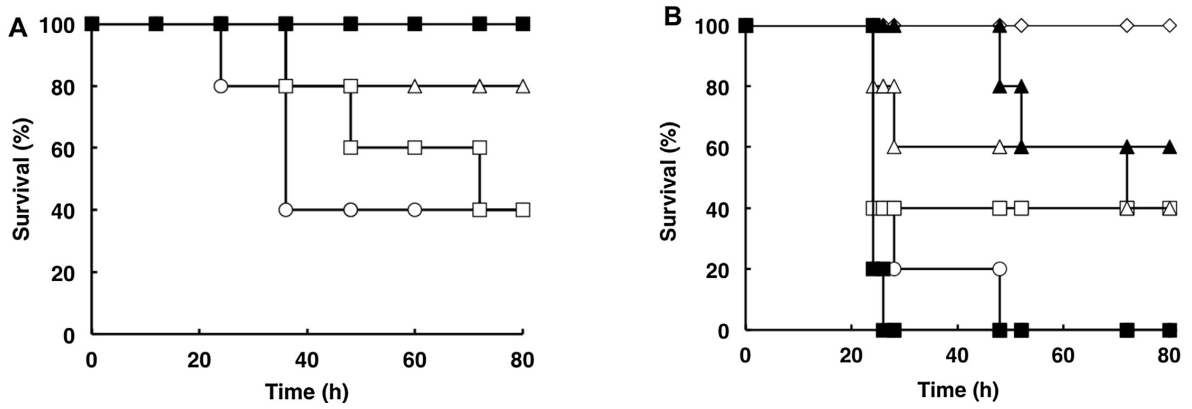

Figure 20. Protective effect of dc-porphyran and F1 on LPS-induced lethality in mice. (A) Mice were intraperitoneally injected with dc-porphyran (□, ■) or F1 (△, ▲) at 10 (□, △) or 100 mg kg^{-1} (■, ▲) 1 h before intraperitoneal injection of LPS (30 mg kg^{-1}). Survival of each group (n = 5) was observed 80 h after LPS injection. (B) Mice were intravenously injected with LPS (20 mg kg^{-1}) together with dc-porphyran (□, ■) or F1 (△, ▲) at 10 (□, △) or 100 mg kg^{-1} (■, ▲) simultaneously. Survival of each group (n = 5) was observed 80 h after LPS injection. ◇, control; ○, LPS alone.

3.2.3.2. Effect of dc-porphyran and F1 on LPS-induced production of TNF-α and NO in mice

Since LPS-induced lethality is mediated by the production of inflammatory mediators such as TNF-*α* and NO, we examined the effect of dc-porphyran and F1 on LPS-induced production of these mediators in mice. Intravenous administration of LPS (20 mg kg^{-1}) induced an increase in serum nitrite levels (27 M) estimated by the Griess assay. Simultaneous injection of F1 reduced the nitrite level significantly to 19 M, while dc-porphyran was almost ineffective (Fig. 21A). An increase in the serum level of TNF-*α* (270 pg mL^{-1}) was also observed, and dc-porphyran or F1 (100 mg kg^{-1}) reduced the value to 200 or 80 pg mL^{-1}, respectively, suggesting that F1 more effectively reduces the TNF-*α* level in mice than does dc-porphyran (Fig. 21B).

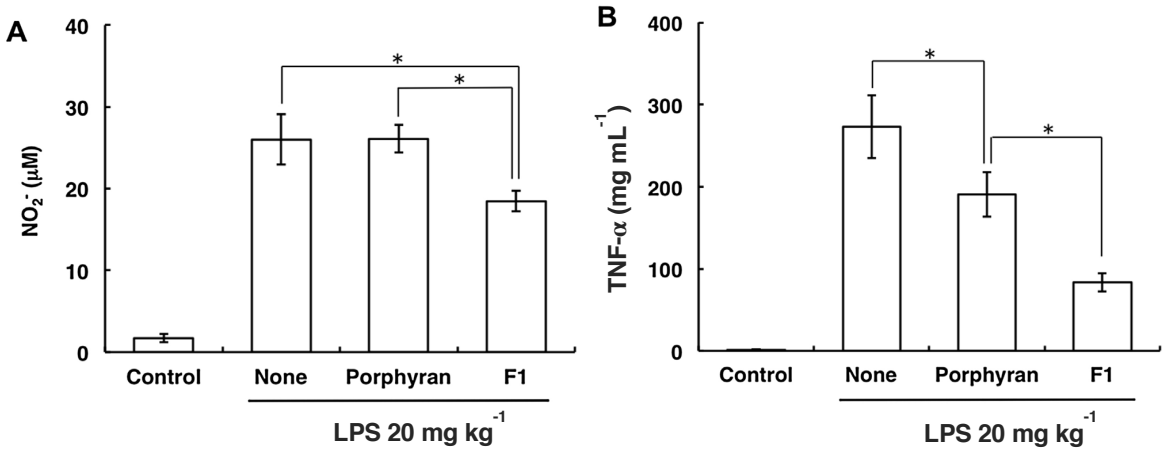

Figure 21. Effects of dc-porphyran and F1 on LPS-induced increase in serum levels of NO and TNF-*α*. Dc-porphyran or F1 (100 mg kg^{-1}) was intravenously injected simultaneously with LPS (20 mg kg^{-1}) and the serum was prepared from mouse blood at 12 h after LPS injection. Control mice were injected with saline alone. The serum nitrite and TNF-*α* levels of each group (n = 3) were determined by Griess assay and ELISA, respectively. Each column represents the mean of triplicate measurements. Each bar represents standard deviation. Asterisks denote significant differences between the experimental groups ($p < 0.05$).

3.2.3.3. Effect of dc-porphyran and F1 on LPS-induced oxidative stress in liver

It has been pointed out that excessive ROS production is related to the progression of sepsis or endotoxin shock,[121,122] and ROS-induced oxidative stress in multiple tissues contributes to high mortality rate.[123,124] Hence, I examined the effect of dc-porphyran and F1 on lipid peroxidation level in the liver by measuring MDA, a marker of oxidative stress. MDA level in the liver homogenate from mice injected only with LPS was increased compared to the level in control mice. Pretreatment with F1 significantly reduced the MDA level, while dc-porphyran had almost no effect (Fig. 22).

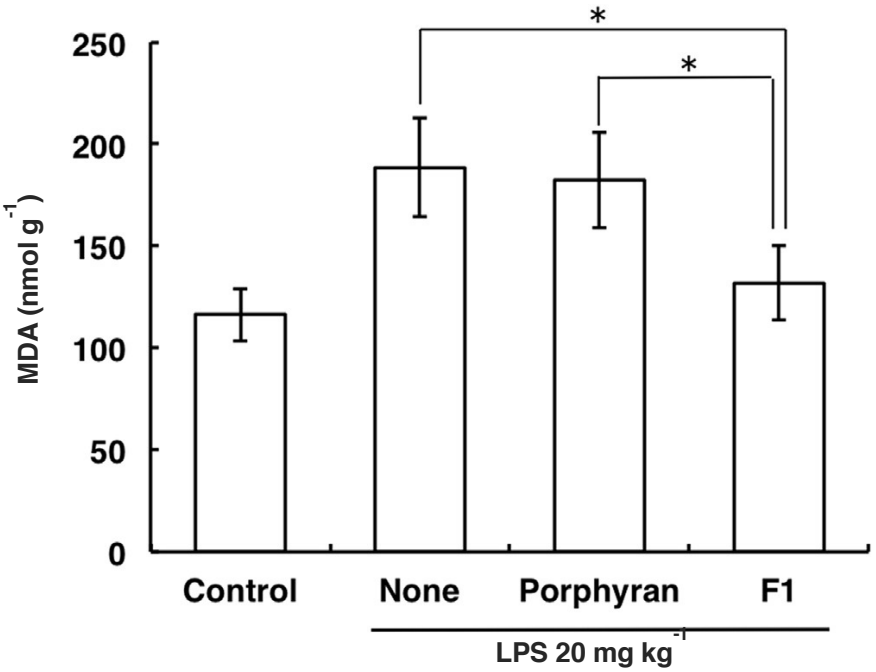

Figure 22. Effects of dc-porphyran and F1 on MDA level in liver. Dc-porphyran or F1 (100 mg kg^{-1}) was intravenously injected simultaneously with LPS (20 mg kg^{-1}). After 12 h, the MDA level of each group (n = 3) in liver was determined. Each column represents the mean of triplicate measurements. Each bar represents standard deviation. Asterisks denote significant differences between the experimental groups ($p < 0.05$).

3.2.3.4. Effect of dc-porphyran or F1 on NO and TNF-α producing activities of peritoneal exudate cells from LPS injected mice

To investigate the effect of i.v. dc-porphyran or F1 on immune-competent cells in mice, peritoneal cells were harvested from mice 12 h after i.v. injection of LPS (20 mg kg^{-1}) together with dc-porphyran or F1 (100 mg kg^{-1}). The cells were incubated in DMEM supplemented with 10% FBS at 37°C for 24 h, and then NO and TNF-α levels in the culture supernatants were measured. As shown in Fig. 23A, NO levels in the culture medium of the peritoneal cells from LPS-injected mice were evidently higher than in those from control mice. Simultaneous injection of dc-porphyran or F1 with LPS resulted in the suppression of the NO levels, and F1 was significantly more effective than dc-porphyran. Consistent with these results, the iNOS mRNA levels in peritoneal macrophages were significantly reduced with F1 injection (Fig. 23B). Similar to NO results, an increase in TNF-α levels in the culture medium of peritoneal cells from LPS-injected mice was observed, and F1 exhibited more effective suppressive effect on increased TNF-α level than did dc-porphyran (Fig. 23C).

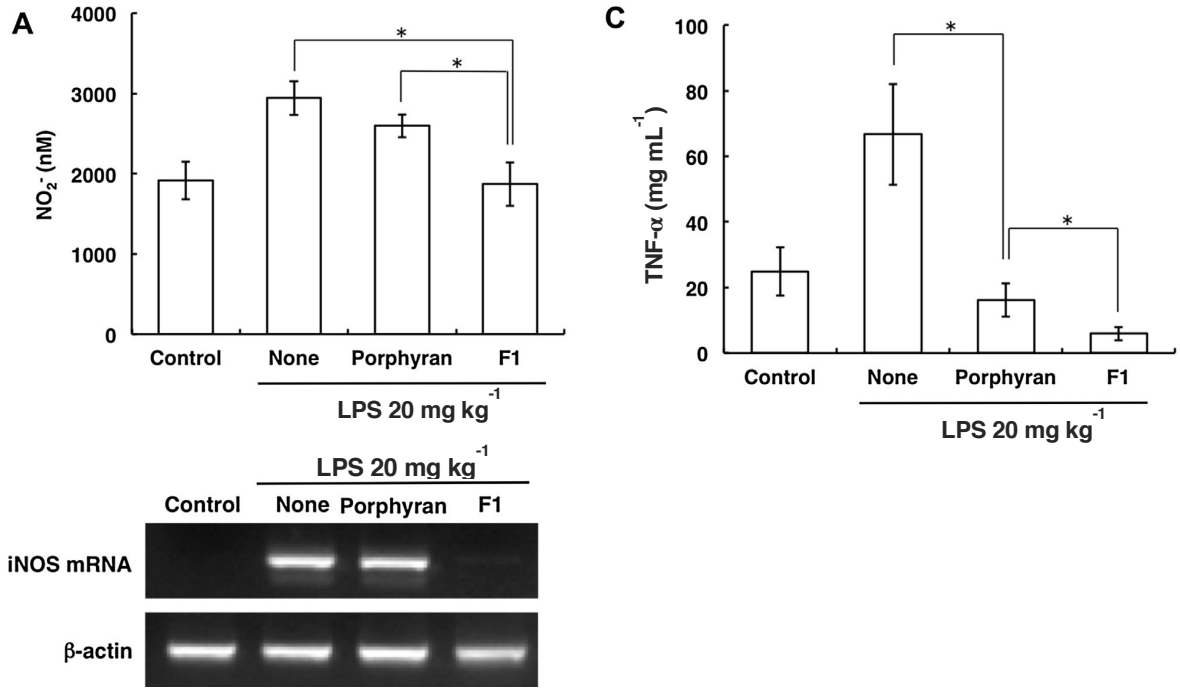

Figure 23. Effects of dc-porphyran and F1 on the activities of peritoneal exudate cells to produce NO and TNF-α. Peritoneal exudate cells were harvested from mice after 12 h intravenous injection of LPS (20 mg kg^{-1}) and dc-porphyran or F1 (100 mg kg^{-1}). The cells were washed twice with PBS by centrifugation and resuspended in DMEM containing 10% FBS, and cultured for 24 h at 37°C in 96-well plates (8×10^4 cells well^{-1}). (A) NO level in the culture medium was measured as described in the text. (B) For the analysis of inducible NO synthase mRNA, 3×10^5 peritoneal macrophages were isolated from mice after 12 h intravenous injection of LPS (20 mg kg^{-1}) together with dc-porphyran or F1 (100 mg kg^{-1}) as described in the text. The cells were subjected to reverse transcription-polymerase chain reaction. (C) TNF-α level in the culture medium was measured by ELISA as described in the text. Each column represents the mean of triplicate measurements. Each bar represents standard deviation. Asterisks denote significant differences between the experimental groups ($p < 0.05$).

3.2.4. Discussion

NO is a gaseous free radical, and plays various physiological roles depending on the sites at which it is generated.[125-130] NO is produced by NO synthase (NOS); endothelial NOS, (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) have been identified so far.[130] In both innate and adaptive immune systems, NO produced by macrophages through iNOS plays a role in anti-infectious immune responses as an important modulator.[129] However, the excess NO produced by iNOS in activated macrophages is involved in numerous severe inflammatory diseases including sepsis and arthritis.[131,132] Since NO particularly induces impaired vascular reactivity and causes pathological changes,[133] the selective inhibition of iNOS expression in macrophages can lead to a certain therapeutic effect for inflammatory diseases. Similar to previous in vitro results on porphyran,[116] it has been reported that fucoidan, a sulfated polysaccharide isolated from brown algae, inhibits NO production and iNOS expression in LPS-activated macrophages.[134] The inhibitory effect of fucoidan on NO production and iNOS expression in other cell types is also reported.[135,136] In contrast to these findings, it has been reported that fucoidan induces NO production in macrophages through a p38 MAP kinase and NF-κB activation mechanism.[137] Although the exact reason for this opposite result is still unclear, it may be attributable to the structural differences between the fucoidans used. In fact, it has been reported that biological activities of fucoidans isolated from nine different sources differed depending on the sources and compositions.[98] Related to these findings, previous study on porphyran showed that the bioactivities of porphyrans isolated from different growth conditions of red alga nori (*P. yezoensis*) were quite different.[117] Namely, a porphyran isolated from discolored nori inhibited NO production in LPS-stimulated RAW264.7 cells, whereas no significant effect was observed for porphyran isolated from normal nori. These findings suggest that the bioactivities of naturally occurring polysaccharides such as fucoidan and porphyran differ depending on the structural features, which can even be altered by the growth conditions of algae. A previous study demonstrated that F1, a fraction purified from porphyran isolated from discolored nori, showed the most potent inhibitory effect on NO production by LPS-stimulated RAW264.7 cells. Since F1 has the lowest molecular size among the four separated fractions, the molecular size is an important structural factor influencing the bioactivities. It have been showed that enzymatic digestion of porphyran resulted in a significant increase in the inhibitory effect on NO production in LPS-stimulated RAW264.7 cells (data not shown). Consistent with previous in vitro results,[117] in the present study,

porphyran isolated from discolored nori, especially F1, was found to be protective against LPS-induced endotoxin shock in mice (Fig. 20A, B). Evidently superior protective effects of F1 against LPS lethality were observed in both i.p. and i.v. injection models compared to that of parental dc-porphyran. To my knowledge, this is the first report showing that porphyran is capable of exhibiting a protective effect on LPS lethality in vivo. After i.v. injection of LPS, NO level in the mouse serum increased significantly. Simultaneously injected F1 suppressed the LPS-induced increase in serum NO level, while the effect of dc-porphyran was almost marginal (Fig. 21A). LPS-induced increase in TNF-α level in the serum was also significantly reduced by F1 (Fig. 21B). TNF-α is known as a major cytokine involved in endotoxin shock,[138] and it induces several cytokines such as IL-1, IL-6 and IL-10, and activates inflammatory cells.[139] Therefore, compounds with the ability to suppress the production of NO and TNF-α can be promising candidates as therapeutic agents for inflammatory diseases and endotoxin shock. In addition, F1 also has ability to reduce the MDA level (Fig. 22). It indicates that F1 may have high potential as an effective therapeutic agent. It has been reported that naringin, a naturally occurring flavonoid with activity similar to F1, suppressed the LPS-induced production of NO and TNF-α, and reduced the LPS lethality in vivo.[140] Hence, suppression of NO and TNF-α production is a common mechanism of porphyran and naringin responsible for protection against endotoxin shock, although there is no structural similarity between porphyran and naringin.

Peritoneal exudate cells (PEC) harvested from mice intravenously injected with LPS showed increased generation of NO and TNF-α compared to PEC of control mice without LPS challenge, suggesting that intravenously injected LPS eventually stimulated PEC. Simultaneous injection of F1 with LPS resulted in significant suppression of the activities of the PEC (Fig. 23A-C). These results suggest that porphyran, especially F1, is capable of suppressing the LPS-induced stimulation of immune-competent cells responsible for the generation of NO and TNF-α in vivo.

Regarding the action mechanism of porphyran, previous in vitro study demonstrated that porphyran inhibited LPS-induced NF-κB activation in RAW264.7 cells through the suppression of phosphorylation and degradation of IκB-α, and subsequent nuclear translocation of the p65 large subunit.[116] These findings suggest that the inhibition of NF-κB activation may be a cellular level action mechanism of porphyran to inhibit iNOS expression in LPS-stimulated macrophages.

The activation status of various transcription factors is influenced by the intracellular redox condition.[141] It is known that activation of NF-κB is induced by various agents including reactive oxygen species,[142] and redox regulation is involved in the activation of NF-κB.[143,143] Antioxidant reagents such as pyrrolidine dithiocarbamate and N-acetylcysteine inhibit the activation of NF-κB,[145,146] and these reagents also suppress the production of NO and TNF-α.[145,147] Recently, it has been suggested that porphyran and fucoidan act as potential antioxidants or radical scavengers.[148,149] In fact, our previous study demonstrated that a porphyran sample showed potent scavenging activity against superoxide anion and hydroxyl radical.[117] Furthermore, fluorescent analysis with a ROS-specific probe revealed that porphyran reduced elevated levels of intracellular ROS in LPS-stimulated RAW264.7 cells, although it was slightly less effective than N-acetylcysteine.[117] Therefore, it is considered that dc-porphyran and F1 inhibited NF-κB activation through an antioxidant property, which may lead to a decrease in TNF-α and NO levels in the serum and eventually to an increase in survival rate of LPS-injected mice.

CHAPTER IV

General Conclusions

HABs are not only a serious environmental issue in Japan, it impact economic on human health, commercial fishery, tourism, monitoring and management all around the world. Therefore, to understand the mechanisms of toxicity of HABs are important for our life. *H. circularisquama* is highly toxic to shellfish and the zooplankton rotifer *Brachionus plicatilis*. A previous study found that *H. circularisquama* has both light-dependent and -independent haemolytic agents, which might be responsible for its toxicity. Detailed analysis of the haemolytic activity of *H. circularisquama* suggested that light-independent haemolytic activity was mediated mainly through intact cells, whereas light-dependent haemolytic activity was mediated by intracellular agents which can be discharged from ruptured cells. Because *H. circularisquama* showed similar toxicity to rotifers regardless of the light conditions, and because ultrasonic ruptured *H. circularisquama* cells showed no significant toxicity to rotifers, it was suggested that live cell-mediated light-independent haemolytic activity is a major factor responsible for the observed toxicity to rotifers. Interestingly, the ultrasonic-ruptured cells of *H. circularisquama* suppressed their own lethal effect on the rotifers. Analysis of samples of the cell contents (supernatant) and cell fragments (precipitate) prepared from the ruptured *H. circularisquama* cells indicated that the cell contents contain inhibitors for the light-independent cell-mediated haemolytic activity, toxins affecting *H. circularisquama* cells themselves, as well as light-dependent haemolytic agents. Ethanol extract prepared from *H. circularisquama*, which is supposed to contain a porphyrin derivative that displays photosensitising haemolytic activity, showed potent toxicity to *C. marina*, *C. antiqua*, and *K. mikimotoi*, as well as to *H. circularisquama* at the concentration range at which no significant toxicity to rotifers was observed. Analysis on a column of Sephadex LH-20 revealed that light-dependent haemolytic activity and inhibitory activity on cell-mediated light-independent haemolytic activity existed in two separate fractions (f-2 and f-3), suggesting that both activities might be derived from common compounds. My results suggest that the photosensitising haemolytic toxin discharged from ruptured *H. circularisquama* cells has a relatively broad spectrum of phytoplankton toxicity, and that physical collapse of *H. circularisquama* cells can lead not only to the disappearance of its own toxicity, but also to mitigation of the effects of other HABs.

C. antiqua isolated in 2010 showed extremely more potent fish-killing activities against red sea bream, Japanese horse mackerel, and blue damselfish than those of *C. marina* isolated in 1985. Chemiluminescence and electron spin resonance (ESR) analyses suggested greater

reactive oxygen species (ROS)-producing activity of *C. antiqua* than that of *C. marina*. Sodium benzoate, a hydroxyl radical scavenger, significantly suppressed the fish-killing activity of *C. antiqua* on blue damselfish. The chlorophyll level in the gill tissue of blue damselfish exposed to flagellate cells increased along with the exposure time, and the cell count of gill-associated *C. antiqua* estimated with chlorophyll level was higher than that of *C. marina*. Therefore the ROS-producing activity and affinity of *Chattonella* cells to the gill surface may be important factors influencing the fish-killing activity of *Chattonella* species. *Chattonella* strains with quite different ichthyotoxicity are useful for shedding light on the toxic mechanism of *Chattonella*.

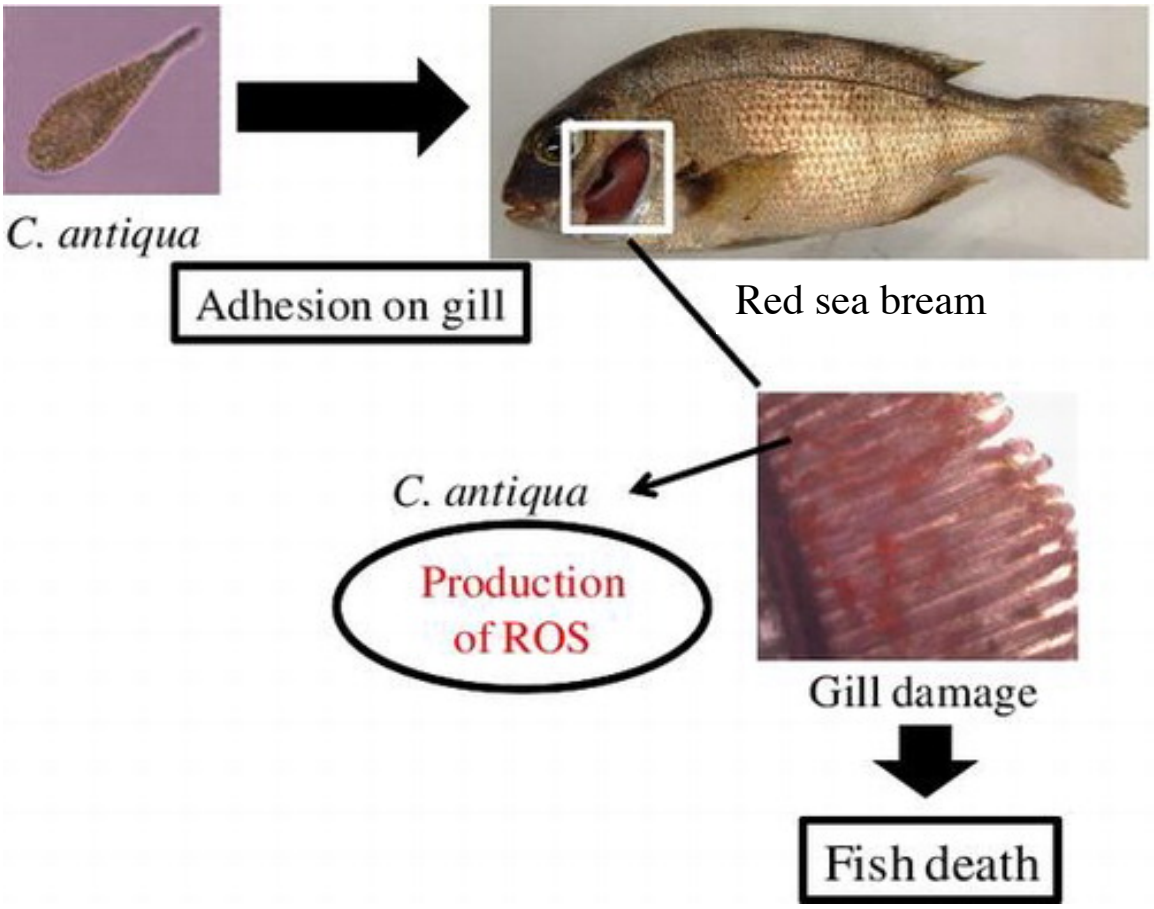

Figure 24. ROS-mediated gill tissue damage by *C. antique* may lead to fish death.

Seaweeds can be considered as promising plants forming one of the important marine living resources of high nutritional value. But sometimes, polysaccharides prepared from marine algae contain contaminants such as polyphenols and endotoxins that may mislead their bona fide biological activities. *F. vesiculosus* fucoidan inhibited the growth of *V. alginolyticus* in a concentration-dependent manner (0-1,000 μg mL^{-1}). However, the antibacterial activity of the fucoidan significantly reduced after methanol-extraction, and the methanol-extract showed a potent antibacterial activity. Although the agents responsible for the toxicities in the methanol-extract could not be fully characterized yet, the extract also showed cytotoxicity to RAW264.7 and U937 cells, and induced apoptotic nuclear morphological changes in U937 cells. These results suggest that the antibacterial activity of the fucoidan is partly due to the methanol-extractable contaminants that can also contribute to the cytotoxicity on RAW264.7 and U937 cells. On the other hand, the activities to induce secretion of nitric oxide and tumor necrosis factor-α from RAW264.7 cells were observed in the fucoidan even after methanol extraction, and the extract had no such activities. It suggest that commercially available fucoidan should be careful when it comes to the studies on the cytotoxic and apoptosis-inducing activities of fucoidan especially the usage of commercially available *F. vesiculosus* fucoidan without further purification.

Porphyran isolated from discolored nori (*P. yezoensis*) (dc-porphyran) and one fraction (F1) purified from dc-porphyran by DEAE-chromatography showed the protective effects on LPS-induced endotoxin shock in mice. Intraperitoneal (i.p.) treatment with dc-porphyran or F1 (100 mg kg^{-1}) 60 min prior to i.p. injection of LPS (30 mg kg^{-1}) completely protected mice from LPS lethality. At 10 mg kg^{-1} concentration, F1 demonstrated more protection than dc-porphyran. Intravenous (i.v.) challenge of LPS, even at 20 mg kg^{-1}, was more lethal than i.p. administration; i.v. injection of F1 (100 mg kg^{-1}) with LPS significantly improved the survival rate. However, i.v. dc-porphyran (100 mg kg^{-1}) produced an even lower survival rate than that of LPS alone. I examined pro-inflammatory mediators such as NO and TNF-α in serum. F1 significantly reduced the levels of these markers. Additionally, F1 significantly decreased the MDA level in the liver, a marker of oxidative stress, while dc-porphyran had almost no effect. Furthermore, F1 significantly decreased the production of TNF-α and NO in peritoneal exudate cells harvested from LPS-challenged mice, while dc-porphyran treatment showed a lesser decrease. It showed that porphyran isolated from discolored nori, especially F1, is capable of suppressing LPS-induced endotoxin shock in vivo. These results suggest that F1 can be a

promising candidate as an effective therapeutic agent for inflammatory diseases.

In this study, I found unique biologically active substances from marine algae which include HABs and seaweeds. The further evaluates of these substances are needed for practical application, whereas this study shed new possibilities of marine algae for utilized in food, biomedical and controlling HAB fields.

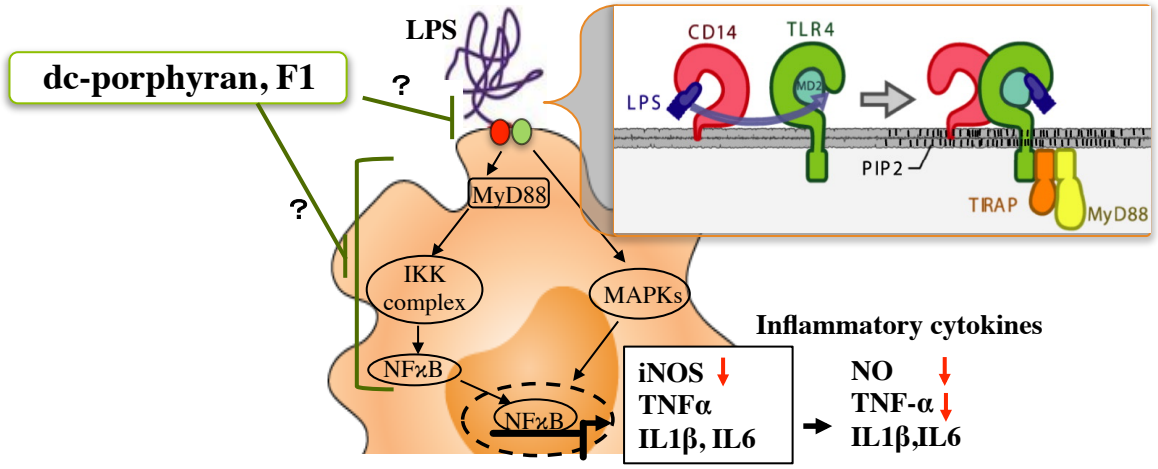

Figure 25. Inhibition of LPS signal pathway by dc-porphyran and F1 suppress the gene expression of inflammatory cytokines.

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