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T. Oda · A. Nakamura · T. Okamoto
A. Ishimatsu · T. Muramatsu

Lectin-induced enhancement of superoxide anion production by red tide phytoplankton

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Abstract *Chattonella marina*, a raphidophycean flagellate, is a highly toxic red tide phytoplankton which causes severe damage to fish farming. Recent studies demonstrated that *Chattonella* spp. continuously release superoxide anions (O_2^-) while they are living. *Heterosigma akashiwo*, another raphidophycean flagellate, also produces O_2^-. In the present study, we found that lectins such as concanavalin A (Con A), wheat germ agglutinin (WGA), and castor bean hemagglutinin (CBH) stimulated *C. marina* and *H. akashiwo* to generate enhanced amounts of O_2^- in a concentration-dependent manner. The lectin-specific sugars potently inhibited the lectin-induced increase of O_2^- production, suggesting that the effects of lectins are mediated mainly through the interaction of these lectins with carbohydrate moiety present on the flagellate cell surface. In contrast to the potent ability of native Con A (tetravalent), succinylated Con A (divalent) showed only a slight stimulative effect on these flagellates. O_2^- production was totally inhibited by treatment with proteinase K for 30 min, without affecting the viabilities of flagellates. These results suggest that cell-surface redox enzymes may be involved in O_2^- production, and such enzymes are responsible for the lectin-stimulation.

Introduction

Blooming of toxic algae, so-called "red tides", frequently causes massive mortalities of cultured fish. Several species of marine plankton have been identified as the causative organisms of red tide (Onoue and Nozawa 1989). *Chattonella marina* is one of the most noxious red tide phytoplankton and is highly toxic to fish, especially to yellowtail, *Seriola quinqueradiata*. In Japan, blooming of *Chattonella* sp. has repeatedly caused severe damage to fish farming. Although the precise mechanism of the toxic action of *Chattonella* sp. remains unclear, recent studies (Ishimatsu et al. 1990, 1991; Tsuchiyama et al. 1992) demonstrated that a decrease in oxygen partial pressure of arterial blood is the earliest physiological disturbance observed in fish after exposure to *C. marina*. In addition, physiological and histological studies of fish exposed to *C. marina* suggested that the blockage of respiratory water flow through the gill lamellae caused by excessive mucus interferes with O_2 transfer, resulting in asphyxia (Matsusato and Kobayashi 1974; Ishimatsu et al. 1996; Hishida et al. 1997). Recently we and other groups have found that *Chattonella* sp. generates reactive oxygen species (ROS) such as O_2^- and H_2O_2 (Shimada et al. 1989, 1991, 1993; Oda et al. 1992a, b, 1994; Tanaka et al. 1992, 1994). Furthermore, our studies using electron spin resonance (ESR) spectroscopy with the spin traps 5, 5-dimethyl-1-pyrroline-*N*-oxide and *N*-*t*-butyl-α-phenylnitrone revealed that *C. marina* generates hydroxyl radicals (·OH) which are known as the most toxic reactive oxygen species (Oda et al. 1992a). Since harmful effects of ROS have been well documented (Babior 1978; Johnson et al. 1981; Halliwell and Gutteridge 1984; Dean 1987; Oda et al. 1989), these results suggest that ROS generated by *Chattonella* sp. may be responsible for gill tissue injury, which eventually causes fish death. Consistent with this hypothesis, our previous results showed that *C. marina* exhibited the ROS-mediated toxic effect on a marine bacterium, *Vibrio alginolyticus* (Oda et al. 1992b). In addition to *Chattonella* sp., it has recently been reported that *Heterosigma akashiwo*, which is also classified as a raphidophycean, produces ROS (Yang et al. 1995). Thus, it is likely that the production of ROS is a common feature of raphidophycean flagellates.

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T. Oda (✉) · A. Nakamura · T. Okamoto · T. Muramatsu
Division of Biochemistry, Faculty of Fisheries,
Nagasaki University, Nagasaki 852, Japan

A. Ishimatsu
Marine Research Institute, Nagasaki University,
Nomozaki, Nagasaki 851-05, Japan

ROS are known to be generated in various biological systems (Boveris et al. 1972; Asada et al. 1974; Thompson et al. 1987; Oda et al. 1989; Sanchez et al. 1993); particularly, phagocytic cells release superoxide anions (O_2^-) into the medium during phagocytosis (Babior 1978) or in response to various membrane reacting reagents such as phorbol myristate acetate (PMA) (Repine et al. 1974) and concanavalin A (Con A) (Goldstein et al. 1977; Nakagawara and Minakami 1979). In addition to animal cells, plant cells also produce ROS in several processes, including ferric reduction during iron uptake (Cakmak et al. 1987), photosynthesis in chloroplast (Asada et al. 1974), and as a defense mechanism against pathogens (Doke 1985; Sanchez et al. 1993). Although the precise mechanism of ROS generation by *Chattonella marina* is still unclear, *C. marina* may have a specific metabolic or enzymatic system that is responsible for the production of ROS. Thus, it was of interest to investigate the effects of several reagents known to modulate the generation of O_2^- in various biological systems, including phagocytic cells.

In the present study, the effect of lectins and various other reagents was examined on the production of O_2^- in *Chattonella marina* and *Heterosigma akashiwo*. We applied a chemiluminescence method using the chemiluminescence probe 2-methyl-6-(*p*-methoxyphenyl)-3, 7-dihydroimidazo[1,2-*a*] pyrazin-3-one (MCLA), which is a *Cypridina* luciferin analog reacting specifically with O_2^- (Lee et al. 1995). The results show that concanavalin A (Con A), wheat germ agglutinin (WGA), and castor bean hemagglutinin (CBH) strongly enhanced the MCLA-dependent chemiluminescence responses by *C. marina* and *H. akashiwo*, and the effects of these lectins were strongly inhibited by lectin-specific sugars.

Materials and methods

Materials

Superoxide dismutase (Cu, Zn-SOD) (3800 U mg^{-1} of protein, from bovine erythrocyte), horseradish peroxidase (100 U mg^{-1} of protein), and dichlorophenyldimethylurea (DCMU) were purchased from Wako Pure Chemical Industry, Co., Ltd., Osaka, Japan. MCLA was obtained from Tokyo Kasei Kogyo, Co., Ltd. Tokyo, Japan. Cytochrome *c* (from horse heart), Con A, succinylated Con A, WGA, and PMA were obtained from Sigma Chemical Co. (St. Louis, Missouri). CBH was purified from castor bean as previously described (Mise et al. 1977). Other chemicals were of the highest grade commercially available. A marine bacterium, *Vibrio alginolyticus*, was isolated from seaweed and maintained in our laboratory as described by Oda et al. (1992b).

Plankton culture

Chattonella marina isolated in Kagoshima in 1985 were generously provided by Kagoshima Prefectural Fisheries Experimental Station, Japan. Axenic clonal culture of this strain was established by repeated-dilution culture and by treatment with antibiotics. An axenic culture of a clonal strain of *Heterosigma akashiwo* (NIES-6) was from the National Institute for Environmental Studies, Environmental Agency, Japan. These flagellates were cultured at 26 °C in sterilized Erd-Schreiber modified (ESM) medium (pH 8.2) under 3000 lx illumination with a cycle of 12 h light and 12 h dark (Shimada et al. 1983; Oda et al. 1992b). Under these conditions, maximum cell concentrations of 5×10^4 cells ml^{-1} for *C. marina* and 6×10^5 cells ml^{-1} for *H. akashiwo* were routinely attained. Unless otherwise noted, flagellates in the exponential growing phase were used (1 to 3.5×10^4 cells ml^{-1} for *C. marina* and 1 to 5×10^5 cells ml^{-1} for *H. akashiwo*) throughout the experiments. All cultivation was done using sterilized instruments. Cells were counted with a hemocytometer.

Chemiluminescence assay

In the chemiluminescence analysis for the detection of O_2^- produced by flagellates we used MCLA as a superoxide-specific chemiluminescent probe as previously described (Lee et al. 1995). MCLA was dissolved in distilled water (1 m*M*) and stored at −80 °C until use. The reaction mixtures typically consisted of 145 µl flagellate cell suspension (1 to 3.5×10^4 cell ml^{-1} for *C. marina* or 1 to 5×10^5 cell ml^{-1} for *H. akashiwo*), 50 µl MCLA solution (20 µ*M*), and 5 µl lectin or other sample solution. The reaction mixtures with ESM medium but without flagellate cells were used as controls. After the addition of MCLA to the flagellate cell suspension, chemiluminescence response was recorded immediately with a luminometer (Model LB 9507, Laboratorium Berthold AG, Wildbad, Germany), using transparent polystyrene tubes.

Preparation of mucus from gill lamellae of yellowtail

Yellowtail, *Seriola quinqueradiata*, weighing 2.5 to 3.0 kg were used for the preparation of gill mucus. They were anesthetized with benzocaine (ethyl *p*-aminobenzoate), and the gills were cleaved of blood before dissection of the gill tissue. The mucus was collected from gill lamellae by scraping with a slide glass. Pooled mucus from nine fish was dialyzed against distilled water for 3 d at 4 °C, and the non-dialyzable part was centrifuged at 4000 ×*g* for 30 min to remove insoluble materials. The supernatant was lyophilized and stored at −80 °C as gill mucus before use.

Results

MCLA-dependent chemiluminescence responses of *Chattonella marina* and *Heterosigma akashiwo*

C. marina and *H. akashiwo* kept in ESM medium were assayed for chemiluminescence immediately after the addition of MCLA (final concentration, 5 µ*M*). MCLA alone in ESM medium gave slight emission as previously reported (Lee et al. 1995). In the absence of MCLA, no significant chemiluminescence response was observed with these flagellate cell suspensions (data not shown). When MCLA was added to *C. marina* or *H. akashiwo* cell suspension, rapid chemiluminescence resposes were observed without a time lag. Nearly maximal chemiluminescence response occurred during the first 30 s of exposure of flagellate cells to MCLA (see Fig. 3). Therefore, the activity of flagellate cells to induce chemiluminescence response was expressed in terms of relative intensity of integrated emission during the first 30 s. As shown in Fig. 1, the chemiluminescence responses induced by *C. marina* and *H. akashiwo* were suppressed by the addition of SOD (100 U ml^{-1}) to background level, which was essentially the same as the luminescence in a MCLA in ESM medium alone. These

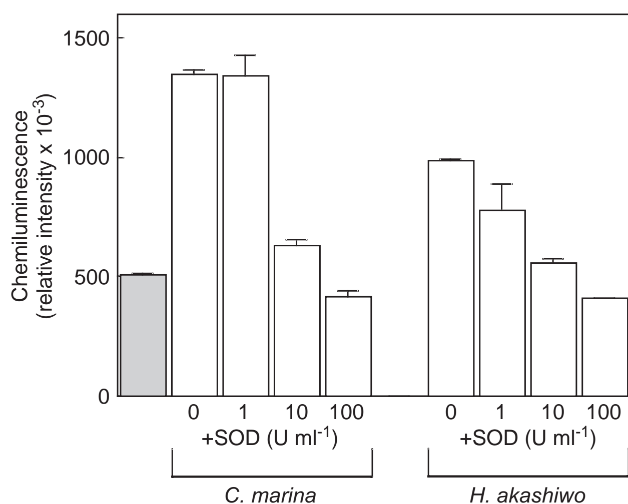

Fig. 1 *Chattonella marina*, *Heterosigma akashiwo*. MCLA-dependent chemiluminescence responses in *C. marina* and *H. akashiwo*. Immediately after the addition of MCLA (final concentration, 5 μ*M*) to flagellate cell suspensions (3.3×10^4 cells ml^{-1} for *C. marina* and 3.6×10^5 cells ml^{-1} for *H. akashiwo*), the chemiluminescence response was measured during the first 30 s at 27 °C in the presence or absence of SOD (1 to 100 U ml^{-1}) (▭). Luminescence of MCLA in ESM medium without flagellate cells is also shown (▇)

results confirm that the luminescence responses were mediated by O_2^- produced by the flagellate cells. The chemiluminescence responses of these flagellates were proportional to cell concentrations, and about 100-fold stronger emission was induced by *C. marina* than by *H. akashiwo* as compared on the basis of cell number (Fig. 2). No significant chemiluminescence was observed in heat-treated flagellate cells (data not shown), similar to the results of ESR analysis (Oda et al. 1992a).

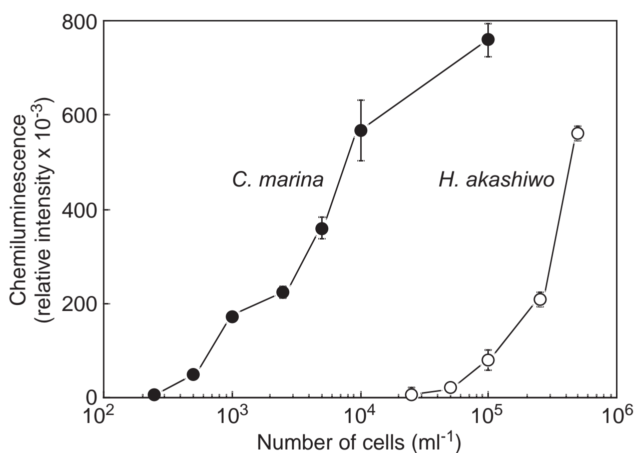

Fig. 2 *Chattonella marina*, *Heterosigma akashiwo*. Relationship between cell concentration and chemiluminescence response in *C. marina* (●) and *H. akashiwo* (○). Chemiluminescences were measured at various concentrations of flagellate cells as described in Fig. 1. Each data point represents the average of duplicate measurements. Luminescence of MCLA in ESM medium alone was subtracted from each value as the background level

Effect of various lectins on chemiluminescence induced by *Chattonella marina* and *Heterosigma akashiwo*

Carbohydrate-binding proteins, called lectins, are well known to bind to carbohydrate moiety on the cell surface in a variety of cell types (Colton et al. 1992; Mody et al. 1995), and induce various cellular processes including the stimulation of O_2^- production in leukocytes (Cohen et al. 1980; Kayashima et al. 1980). As shown in Fig. 3, *C. marina* and *H. akashiwo* showed much stronger chemiluminescence responses, compared to normal levels, after addition of Con A (100 μg ml^{-1}). The enhanced chemiluminescence continued for at least 30 s after Con A addition. The increased chemiluminescence was also reduced by the addition of SOD (100 U ml^{-1}) to the background level (data not shown). In addition to Con A, WGA and CBH also induced enhanced chemiluminescence by these flagellates in a concentration-dependent manner (Fig. 4). However, succinylated Con A (divalent) (Gunther et al. 1973) showed a remarkably weaker stimulating effect on the O_2^- production by *C. marina* and *H. akashiwo* than did native Con A (tetravalent) (Fig. 4).

As shown in Fig. 5, the presence of lectin-specific sugar almost completely abolished the lectin-induced enhancement of O_2^- production by the flagellates, although the efficiency of inhibition was slightly different for each sugar.

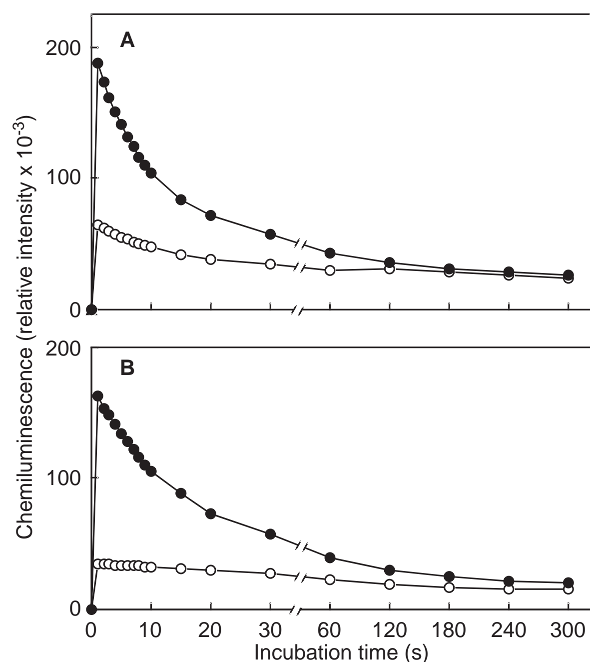

Fig. 3 *Chattonella marina*, *Heterosigma akashiwo*. Effect of Con A on chemiluminescence responses in *C. marina* (**A**) and *H. akashiwo* (**B**). After simultaneous addition of MCLA and Con A to flagellate cell suspension, chemiluminescences were measured as described in "Materials and methods" (○, control; ●, +100 μg Con A ml^{-1})

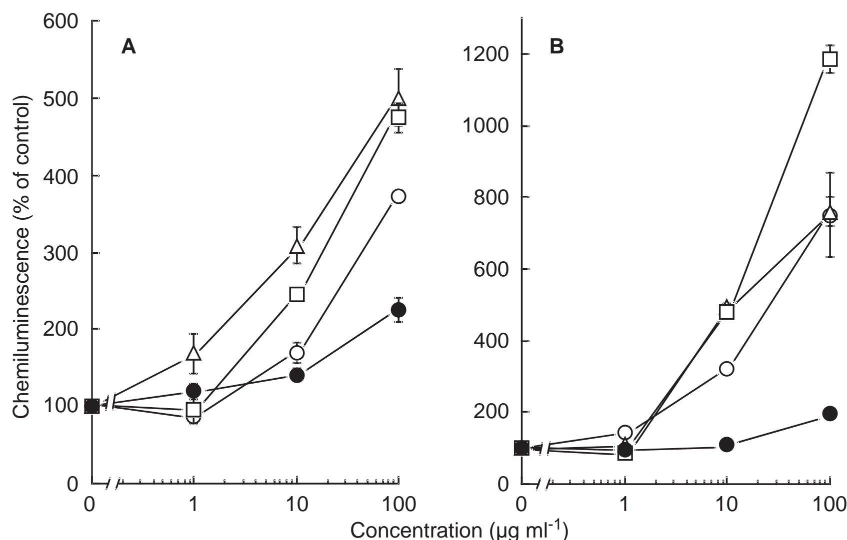

Fig. 4 *Chattonella marina*, *Heterosigma akashiwo*. Concentration-dependent effect of various lectins on chemiluminescence responses in *C. marina* (**A**) and *H. akashiwo* (**B**). After simultaneous addition of MCLA and various concentrations of Con A (○), succinylated Con A (●), WGA (□), or CBH (△) to flagellate cell suspensions, chemiluminescences were measured as described in "Materials and methods". Value of the control (without lectins) was taken as 100%; data represent percent of the ratio of the value in the presence of lectin (1 to 100 µg ml^{-1}) to that in the control. Each data point represents the average of duplicate measurements

Effect of various compounds and marine bacterium on the chemiluminescence responses induced by *Chattonella marina* and *Heterosigma akashiwo*

To gain insight into the mechanism of the O_2^- production by the flagellates, we examined the effect of various inhibitors, and reagents which are known to stimulate O_2^- release by leukocytes. PMA (Repine et al. 1974), cytochalasin B (Nakagawara et al. 1974), and myristic acid (Kakinuma and Minakami 1978), which are known to stimulate O_2^- release by phagocytic cells, did not enhance the chemiluminescence responses by *C. marina* and *H. akashiwo* (Table 1). Furthermore, no significant effect was observed after the addition of the marine bacteria *Vibrio alginolyticus* to the flagellate cultures at a density of 10^7 cells ml^{-1} (Table 1). These results suggest that the O_2^- generating system present in these flagellate cells is not identical to that of mammalian phagocytic cells. The addition of metabolic inhibitors such as potassium cyanide (KCN, an inhibitor of respiration), DCMU (an inhibitor of photosynthesis), and allopurinol (an inhibitor of xanthine oxidase) caused no significant effect on the chemiluminescence responses by these flagellates (Table 1).

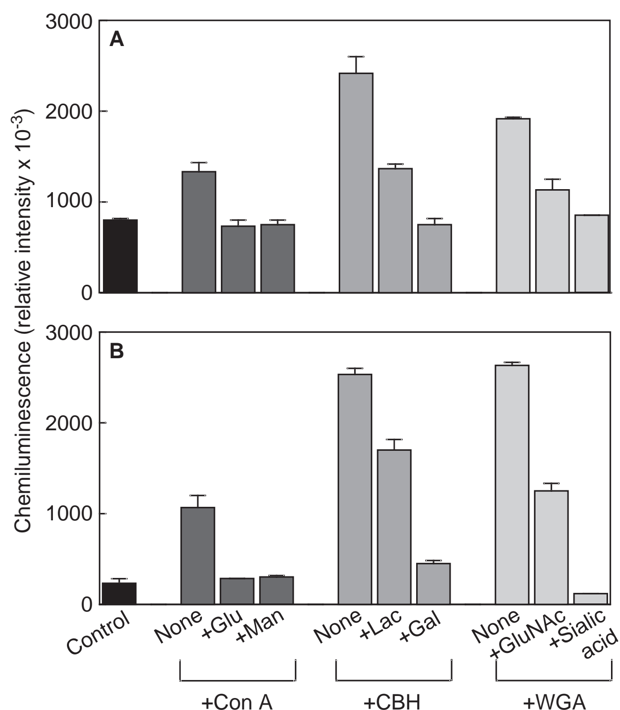

Fig. 5 *Chattonella marina*, *Heterosigma akashiwo*. Effect of lectin-specific sugars on lectin-enhanced chemiluminescence responses in *C. marina* (**A**) and *H. akashiwo* (**B**). After simultaneous addition of MCLA and 10 µg ml^{-1} of Con A (▆), WGA (▆), or CBH (▆) to flagellate cell suspensions containing the given sugars, chemiluminescences were measured as described in "Materials and methods". Final concentrations of sugars used were 100 m*M* for glucose (*Glu*), mannose (*Man*), lactose (*Lac*), *N*-acetylglucosamine (*GluNAc*), and galactose (*Gal*), and 10 m*M* for sialic acid. Luminescence of MCLA in ESM medium alone was subtracted from each value as the background level. Each bar represents the average of duplicate measurements

Effect of mucus from gill lamellae of yellowtail on chemiluminescence induced by *Chattonella marina* and *Heterosigma akashiwo*

It has been reported that *Chattonella antiqua*, when exposed to the mucus from gill lamellae of yellowtail (*Seriola quinqueradiata*), produced increased amounts of O_2^- concomitant with the release of many small particles called mucocysts (Tanaka et al. 1994). As shown in Fig. 6, the addition of 100 µg ml^{-1} mucus prepared from gill lamellae of yellowtail induced enhancement of chemiluminescence by *C. marina* and *H. akashiwo*. The profile of mucus-enhanced chemiluminescence responses was slightly different from the lectin-induced pattern (Fig. 3). Namely, a longer period of peak activity was observed.

Table 1 *Chattonella marina*, *Heterosigma akashiwo*. Effect of different compounds and *Vibrio alginolyticus* on the chemiluminescence responses in *C. marina* and *H. akashiwo*. Chemiluminescence responses in the flagellate cell suspensions were measured in the absence or presence of the indicated concentration of each compound or bacteria, as described in "Materials and methods". Values represent averages ± SE of triplicate determinations (*PMA* phorbol myristate acetate; *KCN* potassium cyanide; *DCMU* dichlorophenyldimethylurea)

Compound added	Chemiluminescence response (% of control)	
	C. marina	*H. akashiwo*
PMA (0.1 μg ml^{-1})	86.3 ± 10.6	103.3 ± 0.7
Cytochalasin B (1 μg ml^{-1})	80.6 ± 0.9	99.2 ± 5.2
Myristic acid (0.1 m*M*)	86.2 ± 0.2	100.0 ± 12.2
KCN (1 m*M*)	111.0 ± 1.1	115.3 ± 10.8
DCMU (10 μ*M*)	86.6 ± 5.6	113.0 ± 3.4
Allopurinol (10 μ*M*)	84.6 ± 1.7	100.8 ± 6.0
Vibrio alginolyticus (10^7 cells ml^{-1})	99.0 ± 15.1	95.3 ± 2.1

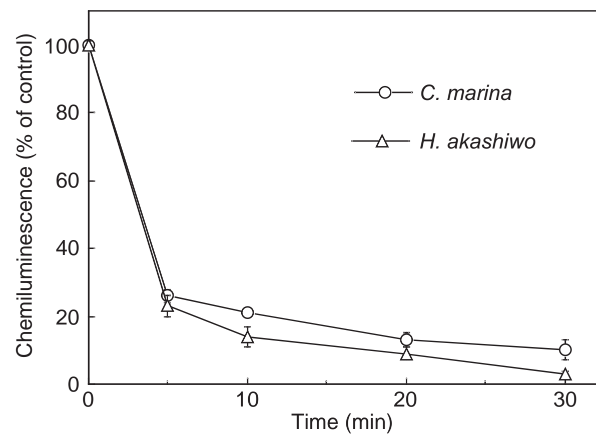

Fig. 7 *Chattonella marina*, *Heterosigma akashiwo*. Effect of proteinase K on chemiluminescence responses in *C. marina* (○) and *H. akashiwo* (△). After the flagellate cells were pretreated with 100 μg ml^{-1} of proteinase K for the indicated period of time at 27 °C, chemiluminescences were measured as described in "Materials and methods". Value of the control (without proteinase K) was taken as 100% data represent percent of the ratio of the value for proteinase K treatment to that in the control. Each data point represents the average of duplicate measurements

Effect of proteinase K on chemiluminescence induced by *Chattonella marina* and *Heterosigma akashiwo*

Previous studies have shown that O_2^- generated by *C. marina* and *H. akashiwo* could be detected by SOD-inhibitable cytochrome *c* reduction assay (Oda et al. 1992a, b; Yang et al. 1995), and that O_2^- can generally be considered membrane-impermeable. Thus, it can be speculated that cell-surface enzymes may be responsible for the production of O_2^-. To examine this point, we used protease proteinase K. As shown in Fig. 7, the chemiluminescences of the flagellates were totally inhibited by treatment with proteinase K (100 μg ml^{-1}) for 30 min. No significant morphological changes were observed during 30 min, and the viabilities of flagellates were not decreased in the presence of 100 μg ml^{-1} of proteinase K, at least during 24 h. Proteinase K is a nonspecific protease; its large size probably prohibits cell permeation. Production of O_2^- by *C. marina* and *H. akashiwo* is thus apparently due to the activity of cell-surface redox enzymes.

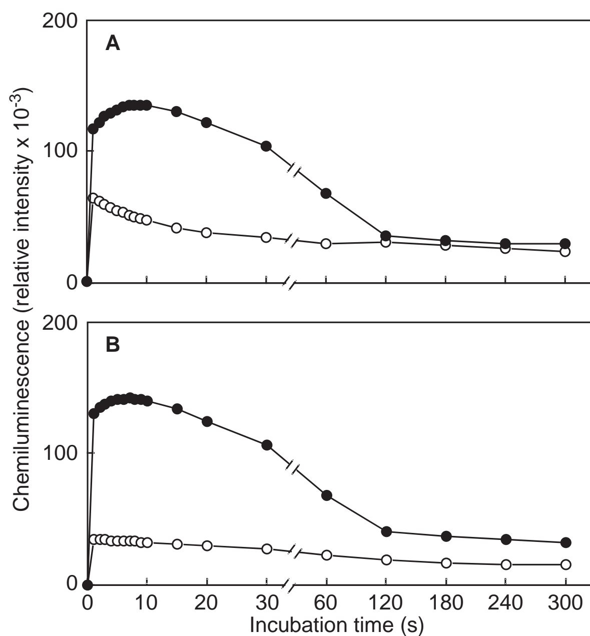

Fig. 6 *Chattonella marina*, *Heterosigma akashiwo*. Effect of mucus from gill lamellae of yellowtail (*Seriola quinqueradiata*) on chemiluminescence responses in *C. marina* (**A**) and *H. akashiwo* (**B**). After simultaneous addition of MCLA and mucus (final concentration, 100 μg ml^{-1}) from gill lamellae of yellowtail to flagellate cell suspensions, chemiluminescences were measured as described in "Materials and methods" (○, control; ●, +mucus)

Discussion

It has been demonstrated that O_2^- is produced in various biological systems (Boveris et al. 1972; Asada et al. 1974; Thompson et al. 1987; Oda et al. 1989; Sanchez et al. 1993), particularly during respiratory burst of phagocytic cells. Here O_2^- is generated by a special NADPH-oxidase system consisting of plasma membrane–associated cytochrome *b*, flavoprotein and several cytosolic components (Segal 1989). This enzyme system is activated and generates O_2^-, when phagocytic cells encounter appropriate soluble or particulate stimuli such as PMA (Repine et al. 1974), fatty acid (Kakinuma and Minakami 1978), lectins (Goldstein et al. 1977; Nakagawara and Minakami 1979; Cohen et al. 1980; Kayashima et al. 1980), other membrane-perturbing agents (Kakinuma et al. 1976; Oda et al. 1986), or immune reactants (Goldstein et al. 1976; Kiyotaki et al. 1978). The ability of the NAD(P)H oxidase found in

plant plasma membranes to generate O_2^- and H_2O_2 has also been shown (Thompson et al. 1987; Vianello and Macrì 1991).

The present study demonstrated that the marine phytoplankton *Chattonella marina* and *Heterosigma akashiwo* produce and release O_2^- under normal growth conditions, as measured by the MCLA-dependent chemiluminescence method, and that greatly increased amounts of O_2^- were released by stimulation with lectins such as Con A, WGA, and CBH, in a concentration-dependent manner (Figs. 3, 4). The chemiluminescences induced by these flagellates were almost completely inhibited by SOD, confirming that the observed luminescence responses were due to the O_2^- released (Fig. 1). Lectin activity is generally considered to be specific for a particular sugar and is competitively inhibited in the presence of the appropriate sugar (Damjanov 1987; Colton et al. 1992; Mody et al. 1995). In agreement with these findings, the specific sugars for each lectin, which have been known to efficiently inhibit the hemagglutinating activity of the lectin (Goldstein and Hayes 1978), acted as potent inhibitors for lectin-induced increase of O_2^- production by *C. marina* and *H. akashiwo* (Fig. 5). Thus, the stimulation of flagellate cells by the lectins seems to be mediated mainly through the interaction of these lectins with carbohydrate moieties on the flagellate cell surface. From the responsiveness of the flagellate cells to Con A, WGA, and CBH, it can also be assumed that at least glucose/mannose-, galactose-, and β-*N*-acetylglucosamine/sialic acid-containing carbohydrate moieties are present on the flagellate cell surface. Regarding the cell surface structure of raphidophycean flagellates, the presence of a glycocalyx on the cell surface of *C. antiqua* and *H. akashiwo* has been demonstrated using electron and light microscopes, although these flagellates are naturally wall-less, and have therefore been called naked plankton (Yokote and Honjo 1985; Yokote et al. 1985).

Histochemical studies revealed that the glycocalyx consists of sulfated and non-sulfated complex carbohydrates together with a neutral carbohydrate–protein complex with Con A-specific sugar residues (α-D-mannosyl and α-D-glucosyl) (Yokote and Honjo 1985; Yokote et al. 1985). This is consistent with our present results in which these flagellates were responsive to Con A stimulation. In contrast to the potent ability of native Con A (tetravalent) to stimulate O_2^- release by the flagellate cells, succinylated Con A (divalent) showed only a slight effect on these flagellates. Similar to our results, it has been reported that native Con A, which can induce cross-linking of surface receptors on the cell surface, but not succinylated Con A, which lacks the cross-linking activity, caused significant O_2^- release from macrophages (Yasaka and Kambara 1979). Therefore, it seems likely that a cross-linking mechanism of lectin receptors on the cell surface is one of the factors triggering the release of O_2^- from macrophages. The cross-linking of lectin receptors may induce membrane perturbation accompanied by the redistribution of receptor molecules on the surface membrane, resulting in the activation of NADPH oxidase. Although the precise mechanism behind flagellate stimulation by lectins is still unclear, the binding of lectin molecules may cause perturbation of cell surface structures. Such changes may activate redox enzymes responding to O_2^- generation as speculated in macrophages. It should be noted, however, that *Chattonella marina* and *Heterosigma akashiwo* generate a certain level of O_2^- under normal growth conditions without addition of specific stimulants or triggers to the flagellate cell suspension (Figs. 1, 2) (Oda et al. 1992a, b, 1994; Shimada et al. 1993; Tanaka et al. 1994). This observation is significantly different from O_2^- generation by phagocytic cells in mammals, which require specific stimulants for the respiratory burst (Nakagawara et al. 1974; Repine et al. 1974; Goldstein et al. 1976, 1977; Kakinuma et al. 1976; Nakagawara and Minakami 1979; Cohen et al. 1980; Oda et al. 1986; Segal 1989; Colton et al. 1992). Although we can not rule out the possibility that these flagellates are responding to unknown stimulants or bacteria which might be present in the cultures, the addition of *Vibrio alginolyticus* did not increase the chemiluminescence responses of these flagellates (Table 1).

It may, therefore, be speculated that the metabolic or enzymatic systems responsible for O_2^- production in *Chattonella marina* and *Heterosigma akashiwo* are more similar to those of higher plant cells than those of animal cells. However, neither the photodynamic reaction nor mitochondrial respiration in the flagellate cells may be involved in O_2^- generation, since 10 μ*M* DCMU and 1 m*M* KCN had almost no effect on the chemiluminescence (Table 1). The lack of effects of *m*-chlorophenylhydrazone (an uncoupler) as well as DCMU and KCN on O_2^- generation by *Chattonella antiqua* has also been reported as measured by SOD-inhibitable reduction of cytochrome *c* (Tanaka et al. 1994). Therefore, we examined the involvement of cell-surface enzymes by using cell-impermeable protease proteinase K. The chemiluminescence responses of *C. marina* and *H. akashiwo* were strongly inhibited by proteinase K treatment without affecting the viabilities of the flagellates, suggesting that cell-surface redox enzymes may be responsible for the O_2^- generation (Fig. 7). This notion is also supported by the recent finding that O_2^- was generated in small particles or verruciform protrusions located on the surface of *C. antiqua* cells (Shimada et al. 1993; Tanaka et al. 1994). In higher plant cells, it has been reported that there are some NADH oxidation activities capable of generating O_2^- and H_2O_2 in plant plasma membranes (Thompson et al. 1987; Vianello and Macrì 1991). Since this NADH oxidase activity was inhibited by the iron-specific chelator Desferal, it has been speculated that the enzyme activity is strictly dependent on the presence of iron ions, which seem essential for the generation of O_2^- (Vianello et al. 1990). Similar to the NADH oxidase activity of the radish plasma membrane, we recently found that the generation of O_2^- by *C. marina* was also inhibited by Desferal (Kawano et al. 1996).

Regarding the toxic mechanisms of *Chattonella* sp. on fish, recent studies demonstrated that a decrease in oxygen partial pressure of arterial blood is the earliest physiological disturbance observed in fish after *Chattonella* sp. exposure (Ishimatsu et al. 1990, 1991; Tsuchiyama et al. 1992). Furthermore, several lines of evidence suggest that excessive mucus on the gill surface, probably induced by *Chattonella* sp., may interfere with O_2 transfer, resulting in asphyxia (Matsusato and Kobayashi 1974; Ishimatsu et al. 1996; Hishida et al. 1997). Oxygen radicals are known to induce mucin secretion from mucus cells in guinea pig gallbladder (LaMont 1989) and rat gastric mucosa (Hiraishi et al. 1991). Thus, it is possible that oxygen radicals produced by *Chattonella* sp. induce excessive mucus secretion on gill lamellae. Shimada et al. (1993) and Tanaka et al. (1994) reported that an increased amount of O_2^- was generated concomitant with the discharge of mucocysts in *C. antiqua*, when the flagellate cells were exposed to mucus from the gill lamellae of yellowtail. Consistent with these findings, the addition of 100 μg ml^{-1} of mucus from gill lamellae of yellowtail caused enhanced chemiluminescence responses in *Chattonella marina* and *Heterosigma akashiwo* (Fig. 6). These results suggest that the release of reactive oxygen might be enhanced when flagellate cells are inhaled into the mouth and contact the surface of the gills. It has been considered that mucus is a complex mixture of materials secreted by goblet cells, and the major mucus component is mucin, composed for the most part of glycoproteins (Alexander and Ingram 1992). Interestingly, several other biochemical compounds including lysozyme, proteolytic enzymes, and agglutinins (lectins or lectin-like molecules) have also been found in fish mucus (Ingram 1980; Fletcher 1981; Ellis 1981). Further studies are required to clarify which compound in mucus is responsible for the stimulation of the flagellate cells.

In conclusion, our results demonstrated that all lectins tested stimulate *Chattonella marina* and *Heterosigma akashiwo* to generate increased amounts of O_2^-, while these flagellates produce a certain level of O_2^- under normal growth conditions without stimulant. The responsiveness of the flagellates to lectin stimulation may provide a clue in understanding the mechanism of oxygen radical generation in the flagellate cells.

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