Generation of superoxide anions by *Chattonella antiqua*: Possible causes for fish death caused by 'Red Tide'

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Abstract. The generation of reactive free radicals by *Chattonella antiqua* ('Red Tide'), which may be a causative factor in fish death, has been demonstrated by electron spin resonance spectroscopy and a microelectrode technique. Electrochemical detection indicates that superoxide anions are produced as a primary product, and undergo further reactions during diffusion in glycocalyx to produce hydrogen, hydroxy-, and carbon-centered radicals which are detected by ESR as secondary products. It is proposed that these reactive radicals are responsible for the destruction of the mucous membranes of fish on exposure to 'Red Tide'.
Key words. Red Tide; *Chattonella antiqua*; superoxide anions; free radical; ESR; microvoltammetry.

Chattonella antiqua is a highly toxic phytoplankton which is harmful to fish, especially in fish farms. The toxic mechanism responsible for mortality of fish exposed to 'Red Tide' remains unknown, but the following important observations from morphological and histochemical studies are reported in recent literature[1-5]. Most of the mucous goblet cells and the mucous coat of fish gill lamellae are destroyed on exposure to 'Red Tide', and there is concomitant shrinkage of the gill epithelium resulting in impaired gas exchange on the gill lamellae. The inhibitory effect of superoxide dismutase (SOD) on the ability of *Chattonella* to reduce ferricytochrome c to ferrocytochrome c[4] indicates that *Chattonella antiqua* generates reactive radicals which are likely to be superoxide anions. In this paper we present further evidence for generation of superoxide anions by *Chattonella*, and propose a mechanism for radical-induced mortality of fish. Superoxide anions (O_2^-) now attract much attention in many fields of biology and medicine, since O_2^- have been detected in various biological systems[6]. They have well documented functions in natural defence mechanisms and have also been proposed as a causative factor in certain cancers[7]. When neutrophils are exposed to stimuli such as phagocytosable particles (bacteria, viruses) or soluble factors (phorbol myristate acetate, calcium ionophores, chemotactic peptides), their oxygen consumption is markedly increased and they produce O_2^- as an initial metabolite, a process which is called a respiratory burst. The active oxygen radicals such as H_2O_2 and OH radicals, produced as secondary products from O_2^-, are thought to act as bactericidal agents[8]. Direct evidence for generation of O_2^- by stimulated neutrophils is provided by electron spin resonance (ESR) using a spin trapping reagent, 5,5-dimethyl-1-pyrroline-1-oxide (DMPO)[9], and more recently by an electrochemical technique[10]. We have now applied both these techniques to ascertain that *Chattonella* generate O_2^- actively.

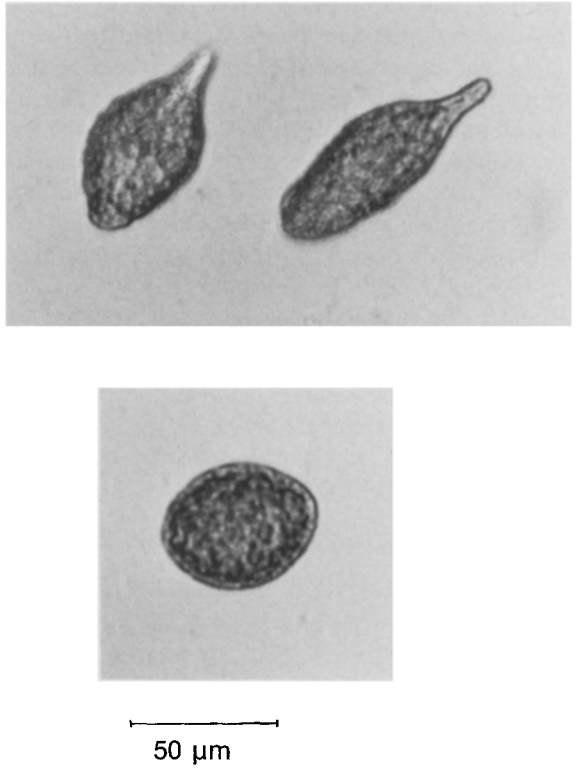

Figure 1. Photomicrographs of *Chattonella* cells. *Chattonella antiqua* was obtained from the Akasio Research Institute of Kagawa Prefecture, and cultured in Erd-Schreiber Modified medium of pH 8.2 with constant illumination at 22 °C. Cultures contained 4-5 × 10^4 cells/ml after 1-2 weeks under these conditions. *Chattonella* cells, when motile, are spindle shaped and propel themselves by the movement of several flagellae (fig. 1a). Treatments such as vigorous shaking or exposure to lower temperatures make them spherical (fig. 1b). The lack of a rigid cell wall makes them too fragile to be separated from culture medium by centrifugation. Formerly *Chattonella* were believed to be 'naked', but recently electron micrographs of *Chattonella* cell sections have demonstrated the presence of a glycocalyx on the cell surface, and histochemical analysis of the structure indicated that this glycocalyx consists of carbohydrates and carbohydrate-protein complexes[11]. The major proportion of Fe(III), which we believe to be important in the mechanism of free radical generation, is in the glycocalyx outside the plasma membrane[12].

The main ESR peaks of the spin adduct from DMPO obtained from spindle shaped *Chattonella* cells (fig. 1) did not coincide with the spin adducts obtained from authentic O_2^- or OH radicals, indicating that the ESR measurement failed to detect either of these radical species (fig. 2). The analysis of the ESR spectrum suggests the existence of two radicals: the H adduct of DMPO (An/G = 16.2 and Ah/G = 22.5)[14], and carbon-centered adducts (An/G = 15.1 an Ah/G = 22.7)[15]. The fact that the ESR signals of spin adducts were diminished on the addition of SOD indicates that O_2^- radicals, although not detected directly, are the active primary product of the cell.

In the electrochemical determination of O_2^-, instability of O_2^-, which rapidly disproportionate to H_2O_2 and O_2^- in aqueous systems, presents us with difficulties. However, the detection of O_2^- can be achieved when the ion is generated at the surface of an electrode where it may be oxidized electrochemically before undergoing further reactions. This procedure was successfully applied to detect O_2^- generated by single stimulated human and porcine neutrophils using a microelectrode[10].

Immediately after a spindle shaped cell was brought into contact with an electrode poised at +0.1 V vs Ag/AgCl (enough positive potential to oxidize O_2^- to O_2), a very large oxidation current burst was observed (fig. 3 B), and the cell became spherical within a few seconds. Such a current burst, however, was not observed when a cell was brought into contact with the electrode poised at −0.2 V vs Ag/AgCl, and the cell kept its spindle shape. An oxidation current burst was partially suppressed when a cell picked from medium to which SOD had been added was brought into contact with the electrode poised at +0.1 V (fig. 3), though the cell became spherical. These results indicate that *Chattonella* cells generate some electrochemically active substances, most probably O_2^-, which produce a current pulse when they contact the electrode poised at a potential to oxidize O_2^- to O_2. From the magnitude and duration of the current pulse, we estimate that O_2^- residing within the cell has an electrical equivalent to 10^{-10} coulomb/cell.

The ESR and electrochemical results show that *Chattonella* generate O_2^- as a primary product. However, because of the high reactivity, O_2^- radicals must undergo

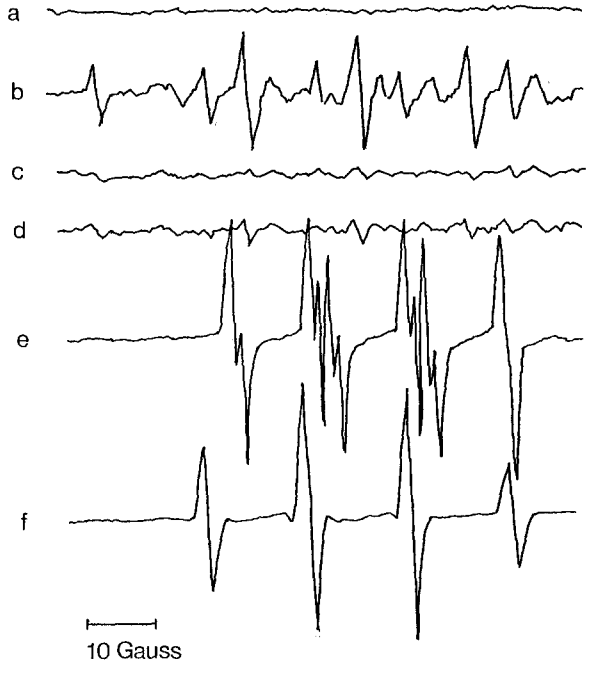

Figure 2. ESR spectra measured using JEX-REIX (JEOL, Japan). a) Spectrum obtained from a cell suspension (3 × 10^4 cells) of *Chattonella* cells in culture medium which were dialyzed for 5–6 h before measurement in artificial sea water prepared using analytical grade chemicals, in order to remove impurities which may affect the result of ESR. No peaks were observed in the absence of spin traping reagent. b) Many peaks appeared immediately after the addition of DMPO to the cell suspension and a slight increase in the height of these peaks was observed with time. c) The addition of SOD and d) catalase caused the elimination of peaks. e) Spin adducts of DMPO with O_2^- generated from a O_2^- generating system comprising cytochrome b558 from porcine neutrophil, cytochrome p450 reductase from rabbit liver and NADPH[13]. f) Spin adducts with OH radicals generated from Fenton's reaction (Fe(II) + H_2O_2).

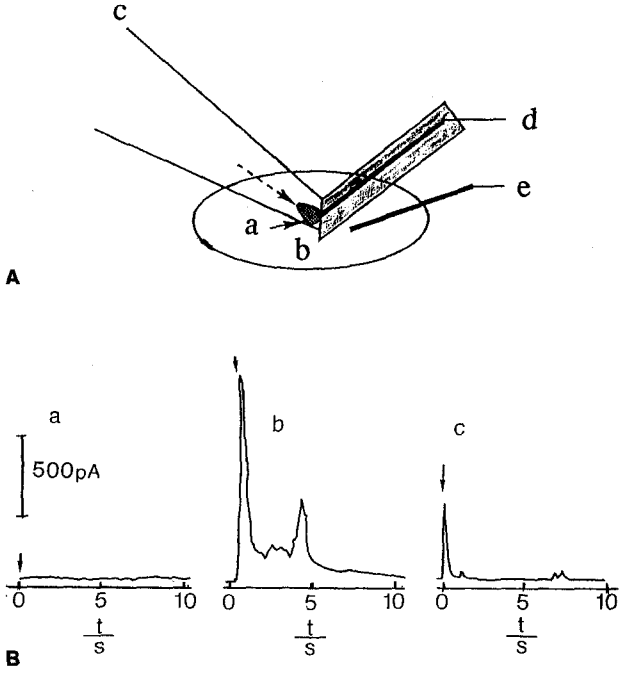

Figure 3. *A* The method for bringing a single cell into contact with the surface of the working electrode made of a 20-μm Pt wire mounted in a glass capillary. A small portion of culture medium (b) was placed on the cover glass under the microscope, and a motile *Chattonella* cell (a) was sucked into the tip of a micropipette (c) connected to microinjector. The working electrode (d) was then placed in position to trap the captured cell in the capillary. The cell in the capillary was then partially ejected to make gentle contact with the electrode surface. The electrode was poised by connecting to a potentiostat Model 972 (Fuso Seisakusho, Japan) at a potential of +0.1 V vs Ag/AgCl (e), which is sufficiently positive to oxidize O_2^-[16]. *B* Examples of current-time profiles obtained with a Pt microelectrode poised at +0.1 V vs Ag/AgCl. Arrows indicate that the electrode was in contact with (a) a spherical non active cell, and (b) a spindle shaped active cell, and (c) an active cell in the presence of SOD.

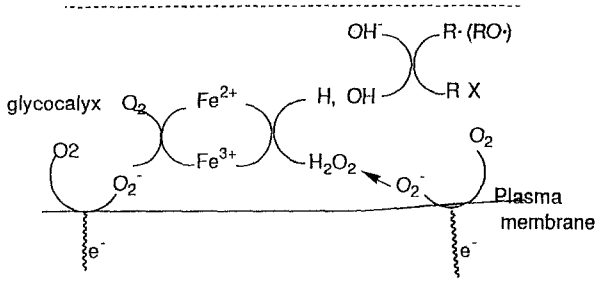

Figure 4. A proposed scheme for reactions occurring in the glycocalyx of *Chattonella* cells when O_2^- is generated in the region of the plasma membrane.

further reactions to produce secondary H−, OH− and carbon-centered radicals. As shown in figure 4, the disproportionation reaction of O_2^- to H_2O_2 and O_2 is in competition with the reduction reaction of Fe(III) to Fe(II) by O_2^- in the glycocalyx. Fe(II) and H_2O_2, produced as secondary products of O_2^-, together produce H and OH radicals when $[H_2O_2] \ll [Fe(II)]$, and an equal amount of H and OH radicals are produced when $[H_2O_2]/[Fe(II)] = 1/10^{14}$. OH radicals are very reactive and undergo further reactions to produce carbon-centered radicals, such as methyl or methoxy radicals, with reaction rates limited by diffusion within the glycocalyx. In the presence of SOD most O_2^- radicals are consumed to produce H_2O_2, and Fe(II) is not produced. Since the enzymatic activity of catalase promotes the reaction $H_2O_2 = H_2O + \frac{1}{2}O_2$, no further reaction occurs in the presence of both enzymes. It is interesting to note that a *Chattonella* cell becomes inactive a few seconds after it comes into contact with the electrode, while the cells lose their activity slowly in the presence of DMPO. Clearly the plankton have a requirement to generate O_2^- for survival, but the 'modus operandi' is unknown. Why and how *Chattonella* produce O_2^- thus remains a challenging problem, and the understanding of the mechanism of O_2^- generation may also help to elucidate the significance of O_2^- in biological systems.

The present results suggest that the generation of active free radicals such as H, OH and carbon-centered radicals, which are the secondary products from O_2^-, may be a dominant factor causing fish death by the 'Red Tide'. Future methods designed to protect fish from damage caused by 'Red Tide' will need to address the detailed chemistry of these primary and secondary active species.

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