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INDUCTION OF SUPEROXIDE DISMUTASES IN *PHOTOBACTERIUM LEIOGNATHI*

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We investigated the induction of Cu,Zn-SOD (bacteriocuprein) and Fe-SOD in *Photobacterium leiognathi* DK-A1 which was isolated from the light organ of the squid, *Droteuthis kensaki*. The induction of superoxide dismutases depended on the addition of paraquat to the medium. Induction of SOD by paraquat was attributed mostly to the bacteriocuprein by measuring of the activities of both SODs by using densitometry of isoelectrofocusing gel. When paraquat was added to the culture at various times in the early log phase of growth, the most efficient induction of the SODs, *which was measured at the time of harvesting the cells (17 hours after inoculation)*, was observed *when paraquat was added* at 60 min after the inoculation. Catalase was not significantly induced by the addition of paraquat or increasing of oxygen concentration.

We developed an assay of SOD by modification of a cytochrome *c*-xanthine oxidase method using a computer equipped absorption spectrophotometer.

KEY WORDS: Bacteriocuprein, induction, paraquat, *Photobacterium leiognathi*, SOD assay, computerized.

INTRODUCTION

Three classes of the superoxide dismutase (SOD) have been reported; those in Fe-SOD, Mn-SOD and Cu,Zn-SOD. The Fe-enzymes have been found mainly in prokaryotes and a few plants, the Mn-enzymes in prokaryotes and eukaryotes and the Cu,Zn-enzymes essentially in eukaryotes.[1] *P. leiognathi* have been reported as first example of a bacterium which contains both Cu,Zn-SOD (bacteriocuprein) and Fe-SOD.[2]

We have shown the induction of the bacteriocuprein by the addition of Cu_2^+ and Zn_2^+ to the growth medium.[3] Puget *et al.*[4] showed that the bacteriocuprein was also induced by oxygen. However, there is no evidence of the inductions of SODs in *P. leiognathi* by paraquat (methyl viologen). Induction of Mn-SOD by the addition of paraquat to the culture of bacterium was first reported by Hassan and Fridovich.[5,6] Paraquat is readily reduced in living cells, as a univalently reduced radical,[7] the radical reacts very rapidly with dioxygen to yield O_2^-.[8] Increased production of O_2^- is a major factor in paraquat toxicity.[6]

In this report, we show the effect of paraquat on the induction of SOD and catalase in *P. leiognathi*. This is a preliminary report on the mechanism of the induction of SODs in *P. leiognathi*.

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MATERIALS AND METHODS

Strain and Culture

P. leiognathi DK-A1 was isolated from the light organ of the squid, *D. kensaki.*[9] Cultures of this strain were grown with vigorous aeration at 23C in a medium which contained 500 ml of artificial seawater, 1 ml of 5.7% K_2HPO_4 solution, 5 ml of 20% NH_4Cl solution, 5 g of Bacto-pepton (DIFCO), 3 g of yeast extract (DIFCO), 3 g of glycerol and 50 ml of 1 M Tris-HCl buffer (pH 7.8) per liter. This nutrition was supplimented by the addition of $CuSO_4$ and $ZnSO_4$ to make 1 mM solution at final concentration. Cells were harvested by centrifugation (4C, 12,000 × g), quickly frozen, and then stored at −40C until sonicated. Thawed cells were suspended (0.25 g/ml) in 50 mM potassium phosphate (pH 7.8), 0.1 mM EDTA and sonicated for 15 min at 9 KHz. Crude extracts were obtained by centrifugation of the sonication at 15,000 × g for 20 min.

Superoxide Dismutase Assay

SOD was assayed by modification of a cytochrome *c*-xanthine oxidase method[10] using a computer equipped absorption specrophotometer on the basis of the equations derived by K. Asada *et al.*[11] This system is composed of a spectrophotometer (HITACHI model 124), *an* AD converter (CONTEC AD-12-16S(98)H), and a microcomputer (NEC PC-9801). Unit value of SOD activity can be calculated immediately after each assay by this system. *All measurements of SOD activity were carried out triplicates by using this system.*

Other methods

Protein concentrations were measured using the method of Lowry *et al.*[12] Analytical isoelectric focusing was carried out by using an ATTO model SJ-1071 isoelectric focusing apparatus in 6% polyacrylamide slab gel containing 2.5% carrier Ampholites (pH 3.5–10.0). The gel was stained for enzymatic activity by the method of Beauchamp and Fridovich.[13] The ratio of the superoxide dismutase activity in the gel was estimated by densitometry. The gel were scanned at 600 nm, with the reference wave length at 800 nm, in a SIMADZU double wavelength chromatoscanner CS-910 type. Catalase activities were measured using the method of Abei.[14]

RESULTS

Induction of SODs by the Addition of Paraquat at the Time of Inoculation

In order to elucidate the time to start induction of the SOD by paraquat, we added the reagent to the medium at the time of inoculation and harvested the cells after 1 hr, 2 hr and 3 hr to measure the SOD activity. The SOD was not induced until 1 hr. After 2 hr the SOD was induced gradually.

We chose the time of cultivation of the cells as 17 hours in order to know the long term effect of paraquat on the induction of SOD of P. leiognathi. *The* P. leiognathi *were grown in media containing different amounts of paraquat for 17 hours and then SOD activities of the crude extracts were measured.* A maximum induction of the SODs was

TABLE I
Estimation of induced SOD by using densitometry on isoelectrofocusing.

	peak height		
	control	paraquat	% induction
Cu,Zn-SOD	1	1.90	190 ± 13.3
Fe-SOD	1.44	1.20	83 ± 0.6

Peak height was expressed as a relative value against the height of Cu,Zn-SOD band on control. Values were averaged to duplicate measurement in densitogram.

obtained at around 0.5 mM of paraquat. With this amount of paraquat, the SODs were induced around 2 times more in the specific activity than in that of the control cultivation.

We chose 0.5 mM of paraquat for further investigation. We measured the ratio of the bacteriocuprein and the Fe-SOD in the crude extracts from the cells of paraquat addition by using densitometer as described in the text. The bacteriocuprein was induced about 2 times more than control values but Fe-SOD does not seem to be inducible by paraquat at this condition (Table I).

Induction of SOD by the Addition of Paraquat after Inoculation

In order to know about the different abilities of the SOD induction of P. Leiognathi *by paraquat in the early growth phase, we added 0.5 mM of paraquat at the times of 0, 30, 60, 90, and 120 min after inoculation and measured the activity of SOD in the cells which were harvested after 17 hours cultivation.*

As shown in Figure 1, the highest ability of induction of SOD of P. leiognathi *by paraquat after 17 hours cultivation was observed at 60 min after inoculation. At 90 min after inoculation, the ability of induction of SOD decreased to a level slightly higher than that of the control value.*

Effect of Paraquat on the Activity of Catalase

The catalase activity was measured for the cells grown with paraquat, 50% oxygen, and paraquat + 50% oxygen. The cells were harvested after 17 hr of cultivation. No significant induction of catalase was observed in the cells grown in each condition.

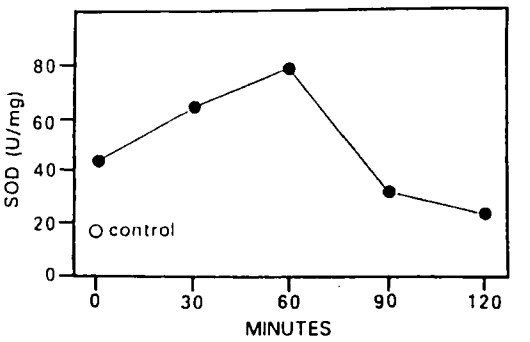

FIGURE 1 Induction of SOD by paraquat at early growth phase. *P. leiognathi* was grown in the medium described in the text. Paraquat was added to make 0.5 mM in final concentration of medium at indicated times *and the cells were harvested and SOD activities were measured at 17 hours after inoculation. The activities of SOD were measured as described in the text.*

DISCUSSION

Although there are few report of evidence that several bacteria contains bacteriocuprein, the physiological function is not clear yet. In this report, we *tried to* show some evidence for the induction mechanism of bacteriocuprein in *P. leiognathi* to elucidate some information on the physiological function of bacteriocuprein. Paraquat induced more bacteriocuprein than Fe-SOD. Therefore bacteriocuprein might be more useful to eliminate O_2^- which was derived by paraquat than by Fe-SOD. This result resembles the case of Mn-SOD induction in *E. coli*,[5-7] in which paraquat induce Mn-SOD but not Fe-SOD.

P. Leiognathi *shows some difference in the ability of the induction of SOD at the time of harvesting the cells (17 hours after inoculation) when the paraquats were added at different times in the early growth phase as shown in Figure 1.*

We speculate three possible mechanisms for this difference in the ability of SOD induction of the cells related to the time of paraquat addition as follows;

1) Activity of paraquat to *generate* O_2^- in the cells might differ according to the time of paraquat addition during cultivation.

2) The cell might produce some O_2^- sensitive target which is essential for the viability of the cells at the specific time of cultivation.

3) The ability of transport of the paraquat into the cells might differ according to the time of cultivation.

Further investigations are required to clarify these possibilities.

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