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Superoxide Dismutase Activity of Lactobacilli

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The presence of superoxide dismutase activity was demonstrated in the dialyzed cell-free extracts of lactobacilli which are considered to be obligatory anaerobes or microaerobes. Specific activities of *Lactobacillus acidophilus* A-28 YIT 0066, *L. casei* C-16 ATCC 7469, *L. arabinosus* ATCC 8014, *L. casei* S-1 YIT 0001 and *L. plantarum* 11-35 YIT 0101 were about 0.2—0.4 units/mg protein, which are much lower than those of *Streptococcus faecalis* and *Escherichia coli*.

Activity was rather constant when *L. acidophilus* A-28 was grown aerobically in the MRS medium containing glucose, mannitol or sorbitol, though the cells grown with mannitol or sorbitol consumed more oxygen than the cells grown with glucose. Activity of the cells grown anaerobically may have constitutively definite activity sufficient for cell survival on exposure to aerobic conditions.

The superoxide dismutase of lactobacilli is not a cupro-zinc enzyme, since it was not inhibited by 5 mM potassium cyanide.

Keywords——superoxide dismutase; lactobacilli; *Lactobacillus acidophilus*; *L. casei*; *L. arabinosus*; *L. plantarum*; oxygen consumption; potassium cyanide-insensitivity

Superoxide dismutase (SOD), an enzyme catalyzing the dismutation of the free radical ion of superoxide,[1] seems to be obligatory in aerobic organisms for defense against oxygen toxicity.[2]

McCord *et al.*[2] reported at first that *Lactobacillus plantarum* did not contain SOD or consume oxygen. Gregory and Fridovich[3] obtained the same results. Yousten *et al.*,[4] however, later reported that *L. plantarum* does possess SOD.

Lactobacilli are anaerobic or microaerobic and do not contain cytochromes and catalase that compose hemoproteins.[5] However, some lactobacilli grow well aerobically and consume oxygen.[6] It is important to determine whether lactobacilli possess some defense mechanisms against oxygen toxicity in order to clarify their aerotolerant nature.

In this communication we show that some lactobacilli possess low but definite SOD activity.

Experimental

Strain——*Lactobacillus acidophilus* A-28 YIT 0066, *L. arabinosus* ATCC 8014, *L. casei* C-16 ATCC 7469, *L. casei* S-1 YIT 0001, *L. plantarum* 11-35 YIT 0101 and *Streptococcus faecalis* ATCC 8043 were kindly provided by Dr. S. Kodaira of Yakult Co., Tokyo. *Escherichia coli* MH-II was from our laboratory collection.

Culture Media——MRS[7] medium was used for the cultivation of lactobacilli and *Str. faecalis*. Ingredients of this medium are as follows: 10 g polypeptone (Wako), 10 g meat extract (Wako), 5 g yeast extract (Wako), 5 g sodium acetate, 2 g ammonium citrate, dibasic, 1 g Tween 80 (Wako), 2 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 5H_2O$, 0.05 g $MnSO_4 \cdot nH_2O$ and 20 g carbon source. D-Glucose (Wako), D-mannitol (Wako) or D-sorbitol (Nakarai) was used as a carbon source. *E. coli* was grown in a nutrient broth.

Culture——The cells were precultured at 30°C for 48 h on a slant medium. One loopful of the cells was inoculated into a Sakaguchi flask containing 150 ml of the medium and cultured aerobically at 30°C under reciprocal shaking (110 osci./min). *L. acidophilus* was also cultured anaerobically. The cells were precultured in a test tube (3 ml of medium) filled with nitrogen gas then inoculated into an Erlenmeyer flask containing 300 ml of the medium. The flask was bubbled through with nitrogen gas until air had been completely replaced, and incubated at 30°C for 40 h under stirring by a magnetic stirrer.

The cultures were harvested in the early stationary phase by centrifugation at $13000 \times g$ for 15 min at 4°C.

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Measurement of Oxygen Consumption——Oxygen consumption of the cells was measured by the conventional manometric procedure at 30°C. The main compartment of a Warburg flask contained 1.0 ml of the cell suspension (10—50 mg dry weight) and 1.0 ml of 0.1 M phosphate buffer (pH 7.0). The side arm of the flask contained 0.5 ml of 0.25 M glucose solution, and a filter paper soaked in 0.2 ml of 20% KOH was fitted in the central well in order to absorb carbon dioxide. After thermoequilibration, substrate solution was poured into the main part of the flask and the manometer was read at periodic intervals for more than 30 min.

Measurement of Dry Weight of Cells——Aliquots of cell suspensions were dried at 110°C for 20 h and the dryweight of the cells was measured.

Preparation of Dialyzed Cell-Free Extract——Harvested cells were washed with 0.05 M carbonate buffer (pH 10.2) and suspended in a small volume of this buffer. The cells were disrupted in a Braun cell homogenizer with chilling under carbon dioxide gas. Homogenates were centrifugated at 20000×*g* for 30 min at 4°C in order to remove intact cells and cell debris. The cell-free extracts thus obtained were dialyzed against 0.05 M carbonate buffer (pH 10.2) for 18 h.

Assay of Superoxide Dismutase Activity——Activity of superoxide dismutase (SOD) in the cell-free extract was assayed by the method of Imanari *et al.*[8] This method is based on the fact that SOD inhibits the reduction of nitroblue tetrazolium (NBT) by O_2^- generated by the action of xanthine oxidase. The reaction mixture contained 0.1 ml of 3 mM xanthine, 0.1 ml of 0.75 mM NBT, 0.1 ml of 3 mM EDTA, 0.1 ml of 1.5 mg/ml bovine serum albumin, 0.1 ml of dialyzed cell-free extract and 2.4 ml of 0.05 M carbonate buffer (pH 10.2). After preincubation for 10 min at 25°C, 0.1 ml of xanthine oxidase (adequately diluted) was added and the mixture was incubated for 20 min at 25°C. The reaction was stopped by the addition of 0.1 ml of 6 mM $CuCl_2$ and the absorbance of this solution at 560 nm was measured and compared with that of the control, to which the cell-free extract had not been added. One unit of SOD is defined as that quantity of the enzyme which would cause 50% inhibition of NBT reduction as described by Imanari *et al.*[8] Water that had been redistilled in glassware was used throughout in order to avoid interference on enzyme actions by contaminating metal ions.

Determination of Protein Content——Protein content of the samples was determined by the method of Lowry *et al.*[9]

Chemicals——Xanthine was purchased from Kojin Co., Tokyo, NBT from Nakarai Co., Kyoto, and xanthine oxidase (from cow milk) from Boehringer Co., Germany. Bovine serum albumin used was "Albumin, Bovine Serum Cryst." from Armour Co., U.S.A. Other chemicals were of reagent grade.

Results

Superoxide Dismutase Activity in the Dialyzed Cell-Free Extract of *L. acidophilus* A-28

The activity of SOD in the dialyzed cell-free extract of *L. acidophilus* A-28 was assayed (Table I). The absorbance of the control, which consisted of the complete system but without the cell-free extract, was 0.216. When xanthine, NBT or xanthine oxidase was omitted from the reaction mixture, the absorbance at 560 nm was negligible. The addition of dialyzed cell-free extract to the reaction mixture inhibited the xanthine oxidase-dependent reduction

Table I. Superoxide Dismutase Activity of *Lactobacillus acidophilus* A-28

Reaction mixture	Cell-free ext. (ml)	Absorbance at 560 nm	Inhibition (%)
Complete	—	0.216	0
-Xanthine	—	0	—
-Nitroblue tetrazolium	—	0	—
-Xanthine oxidase	—	0	—
Complete	0.2	0.210	2.8
Complete	0.3	0.113	47.7
Complete	0.4	0.077	64.4
Complete	0.5	0.034	84.3

Activity of superoxide dismutase in the dialyzed cell-free extract was assayed by the method of Imanari *et al.*[8] The reaction mixture contained 0.1 ml of 3 mM xanthine, 0.1 ml of 0.75 mM nitroblue tetrazolium, 0.1 ml of 3 mM EDTA, 0.1 ml of 1.5 mg/ml bovine serum albumin, dialyzed cell-free extract (3.45 mg/ml of protein) and 0.05 M carbonate buffer (pH 10.2) (total 2.8 ml). After preincubation for 10 min at 25°C, 0.1 ml of xanthine oxidase (adequately diluted) was added and the mixture was incubated for 20 min at 25°C. The reaction was stopped by the addition of 0.1 ml of 6 mM $CuCl_2$ and the absorbance of this solution at 560 nm was measured. When the cell-free extract was added, the absorbance of the extract itself was subtracted.

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of NBT. This inhibition becomes greater with increasing amount of cell-free extract. The addition of 0.5 ml (1.73 mg protein) of cell-free extract inhibited 84% of the NBT reduction of the control.

When the cell-free extract was added after being heated in a boiling water bath for 5, 15 or 25 min, the inhibition of NBT reduction decreased gradually (Table II). These results suggested that the cell-free extract of *L. acidophilus* contains heat-labile superoxide dismutating activity.

TABLE II. Heat Inactivation of Superoxide Dismutase

Cell-free extract (ml)	Absorbance at 560 nm
0	0.234
0.4 (not heated)	0.070
0.4 (heated for 5 min)	0.090
0.4 (heated for 15 min)	0.112
0.4 (heated for 25 min)	0.256

Dialyzed cell-free extract (3.40 mg/ml protein) was heated in a boiling water bath before addition.

TABLE III. Specific Activities of Superoxide Dismutase in Cell-Free Extracts of Aerobically Grown Lactobacilli

Organisms	Specific activity (units/mg protein)
L. acidophilus A-28	0.29±0.04(4)
L. casei C-16	0.32±0.05(3)
L. arabinosus	0.29±0.01(3)
L. casei S-1	0.41(1)
L. plantarum 11-35	0.22(1)
Str. faecalis	55.9±7.2(4)
E. coli MH-II	49.1±9.1(3)

Lactobacilli and *Str. faecalis* were grown in MRS medium. *E. coli* was grown in nutrient broth. Figures in parentheses are numbers of experiments. One unit is defined as that quantity of cell-free extract which would cause 50% inhibition of NBT reduction.

Superoxide Dismutase Activity of Lactobacilli

Specific activities of SOD of several lactobacilli were determined (Table III). All of the lactobacilli used here exhibited low but definite activity of 0.2—0.4 units/mg protein. The SOD activities of *Str. faecalis* and *E. coli* were much higher than those of lactobacilli.

Effect of Potassium Cyanide on the Superoxide Dismutase Activity of *L. acidophilus*

If potassium cyanide inhibits the SOD activity of *L. acidophilus*, addition of potassium cyanide to the reaction mixture will cause an increase of NBT reduction. As shown in Table IV, the decrease of NBT reduction by the cell-free extract (0.201—0.069) was not reversed by the addition of 5 mM potassium cyanide (0.073).

Oxygen Consumption and SOD Activity of the Cells grown in MRS Glucose, Mannitol or Sorbitol Medium

Preliminary studies revealed that *L. acidophilus* grown in a polyol (mannitol or sorbitol) medium consumed more oxygen than the cells grown in a hexose (glucose, mannose, galactose or fructose) medium.[6d] SOD activity was compared among the cells grown in MRS medium containing glucose, mannitol or sorbitol as a carbon source. As shown in Table V, the cells grown in mannitol medium and sorbitol medium consumed about 1.8 times more oxygen than

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TABLE IV. Effects of Potassium Cyanide on Superoxide Dismutase Activity

Cell-free ext. (ml)	KCN (M)	Absorbance at 560 nm
—	—	0.201
0.4	—	0.069
0.4	1×10^{-4}	0.079
0.4	1×10^{-3}	0.073
0.4	5×10^{-3}	0.073

Dialyzed cell-free extract contained 3.24 mg/ml protein.

TABLE V. Superoxide Dismutase Activity of *L. acidophilus* A-28 grown in MRS Medium with Glucose, Mannitol or Sorbitol

Cells grown in	Oxygen consumption (O_2 nmol/min/mg dry cells)	Specific activity[a] (units/mg protein)
Glucose medium	8.08±0.62(4)[b]	0.292±0.043(4)
Mannitol medium	14.55±0.45(3)	0.248±0.039(3)
Sorbitol medium	14.06±0.43(5)	0.270±0.022(5)

a) One unit is defined as in Table III.
b) Figures in parentheses are numbers of experiments.

TABLE VI. Superoxide Dismutase Activity of *L. acidophilus* A-28 grown in a MRS Glucose Medium under Aerobic or Anaerobic Conditions

Cells	Specific activity (units/mg protein)
Anaerobically grown cells	0.310±0.017(3)
Aerobically grown cells	0.292±0.043(4)

One unit is defined as in Table III.

the cells grown in glucose medium. There was no difference in SOD activity among the cells grown in media containing other carbon sources.

Superoxide Dismutase Activity of *L. acidophilus* grown anaerobically

L. acidophilus A-28 grown anaerobically in the MRS glucose medium exhibited about the same SOD activity as the cells grown aerobically (Table VI).

Discussion

There has been controversy about the presence of SOD in lactobacilli. McCord *et al.*[2] and Gregory and Fridovich[3] were not able to demonstrate SOD activity or oxygen consumption in *L. plantarum*. Gregory *et al.*[10] also reported that 7 strains of *L. acidophilus* were SOD-negative (except for one strain). In the view of Gregory *et al.*, lactobacilli can not generate O_2^- or H_2O_2 by reduction of oxygen and therefore would need no enzymatic defenses against these intermediates.

Yousten *et al.*,[4] however, found that both catalase-negative and -positive *L. plantarum* consume oxygen and have SOD activity. Oxygen consumption of lactobacilli has been well documented by Strittmatter[6a] and Brown and Van Demark.[6b] We also reported aerobic growth and oxygen consumption.[6c,d] In addition, NADH oxidase,[6a,b,11] NADPH oxidase,[6b] NADH peroxidase[4,6b,12] and NADPH peroxidase[6b] have been found in the cell-free extracts of

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lactobacilli. These reports suggest that O_2^- or H_2O_2 must have been formed in the cytosol of aerobically grown lactobacilli, and accordingly they should have some defense systems against oxygen toxicity.

It this work, we found that the dialyzed cell-free extract of lactobacilli contains SOD activity. Specific activity was about 0.2—0.4 units/mg protein, almost the same as that of an obligate anaerobe, *Bacterioides melaninogenicus*[10] or *Clostridium pasteurianum* ATCC 6013,[13] though it is much lower than those of some aerobes,[14] and facultative anaerobes.[15] SOD activity of *L. acidophilus* was rather constant, independent of the availability of oxygen or the activity of oxygen consumption. These results seem to indicate that lactobacilli may constitutively have definite activity sufficient to permit their survival upon sudden exposure to oxygen. Generally, three different kinds of SOD (cupro-zinc, manganic and ferric enzyme) are known.[16] SOD of lactobacilli is not a cupro-zinc enzyme since it was not inhibited by 5 mM potassium cyanide. Lactobacilli are very interesting organisms from the evolutionary viewpoint[17] in that some obligate anaerobes became tolerant to oxygen and finally became able to utilize oxygen as an effective electron acceptor.

Almost equal activity was obtained when SOD activity was measured by spectrophotometry according to McCord and Fridovich[1] though the results are not shown here. The method of Imanari *et al.*[8] used here was more quantitative and reproducible, however.

Recently Goetz *et al.*[18] found a non-proteinaceous compound that mimics SOD activity in the cell-free extracts of *L. plantarum*. This activity in the cell-free extract was greatly decreased after dialysis but remained after removal of protein by heat or acid denaturation.

In this work, however, SOD activity was found in the dialyzed cell-free extracts of lactobacilli. This activity was inactivated on heating. In order to clarify the aerotolerant nature of lactobacilli, further work is required.

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References and Notes

1) J.M. McCord and I. Fridovich, *J. Biol. Chem.*, **244**, 6049 (1969).
2) J.M. McCord, B.B. Keele, Jr. and I. Fridovich, *Proc. Natl. Acad. Sci.* (U.S.A.), **68**, 1024 (1971).
3) E.M. Gregory and I. Fridovich, *J. Bacteriol.*, **117**, 166 (1974).
4) A.A. Yousten, J.L. Johnson, and M. Salin, *J. Bacteriol.*, **123**, 242 (1975).
5) R.S. Breed, E.G.D. Murray, and N.R. Smith, "Bergey's Manual of Determinative Bacteriology," 8th ed., The Williams and Wilkins Company, Baltimore, 1971, p. 576.
6) *a*) C.F. Strittmatter, *J. Biol. Chem.*, **234**, 2789 (1959); *b*) J.P. Brown and P.J. Van Demark, *Can. J. Microbiol.*, **14**, 829 (1968); *c*) Y. Iwamoto and I. Mifuchi, *Igaku-to-Seibutsugaku*, **87**, 139 (1973); *d*) Y. Iwamoto, K. Baba, and I. Mifuchi, *Yakugaku Zasshi*, **99**, 354 (1979).
7) J.C. De Man, M. Rogosa, and M.E. Sharpe, *J. Appl. Bacteriol.*, **23**, 130 (1960).
8) T. Imanari, M. Hirota, M. Miyazaki, K. Hayakawa, and Z. Tamura, *Igaku-no-ayumi*, **101**, 496 (1977).
9) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
10) E.M. Gregory, W.E.C. Moore, and L.V. Holdeman, *Appl. Environ. Microbiol.*, **35**, 988 (1978).
11) S. Fukui, *Agric. Biol. Chem.* (Tokyo), **25**, 876 (1961); Y. Iwamoto, K. Baba, and I. Mifuchi, *Yakugaku Zasshi*, **99**, 794 (1979).
12) S. Mizushima and K. Kitahara, *J. Gen. Appl. Microbiol.*, **8**, 56 (1962).
13) J. Hewitt and J.G. Morris, *FEBS Lett.*, **50**, 315 (1975).
14) Y. Chikata, E. Kusunose, K. Ichikawa, and M. Kusunose, *Osaka City Medical Journal*, **21**, 127 (1975).
15) R.N. Costilow and B.B. Keele, Jr., *J. Bacteriol.*, **111**, 628 (1972); O.P. Daily, R.M. Debell, and S.W. Joseph, *J. Bacteriol.*, **134**, 375 (1978); K. Yano and H. Nishie, *J. Gen. Microbiol.*, **24**, 333 (1978).
16) I. Fridovich, *Ann. Rev. Biochem.*, **44**, 147 (1975); K. Asada and S. Kanematsu, "Evolution of Protein Molecules," ed. by H. Matsubara and T. Yamanaka, Japan Scientific Societies Press, Tokyo, 1978, p. 361.
17) J.W. Shopf, *Scientific American*, **239**, 84 (1978).
18) F. Goetz, E.F. Elstner, B. Sedewitz, and E. Lengfelder, *Arch. Microbiol.*, **125**, 215 (1980).

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