Superoxide Dismutase Activity in Some Strains of Lactobacilli: Induction by Manganese

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Dialyzed cell-free extract of lactobacilli was found to contain superoxide dismutase activity by using a test system in which superoxide ion is generated by xanthine oxidase. The specific activities of *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus murinus* ATCC 35020, *Lactobacillus acidophilus* CRL 358, *Lactobacillus plantarum* ATCC 8014, *Lactobacillus casei* CRL 431, *Lactobacillus plantarum* CRL 353, *Lactobacillus fermentum* ATCC 9338, *Lactobacillus buchneri* NCDO 110, and *Lactobacillus fermentum* CRL 251 were between 0.06 and 0.43 U/mg protein. The presence of superoxide dismutase activity was demonstrated when the strains were grown in media containing Mn^{2+} ions. Superoxide dismutase of lactobacilli may be an Mn enzyme since it was not inhibited by either cyanide or azide ions. However, the cell-free extract of *Lactobacillus murinus* ATCC 35020 contains superoxide dismutase activity sensitive to both ions.

Keywords lactobacilli; superoxide dismutase; respiratory enzyme; potassium cyanide insensitivity

Superoxide dismutase (SOD) has been studied extensively in recent years. This enzyme catalyzes the dismutation of univalently reduced oxygen formed in many biological oxidations and is assumed to play an important role in aerobic organisms for defense against the deleterious action of the superoxide radical.[1)]

Lactobacilli can be microaerobic or anaerobic and do not contain either cytochromes or catalase. However, some strains grow well under aerobic conditions and consume oxygen.[2)] McCord and Fridovich[3)] postulated that oxygen uptake by aerobically-living organisms requires the presence of SOD. It is therefore important to determine whether lactobacilli possess some defense mechanism against oxygen toxicity in order to clarify their aerotolerant nature. McCord *et al.*[4)] and Gregory and Fridovich[5)] reported that *Lactobacillus plantarum* did not contain SOD. Later, Yousten *et al.*,[6)] and Iwamoto and Mifuchi[7)] respectively reported that *L. plantarum* and some lactobacilli do possess SOD. In order to investigate the reason for these discrepancies we reexamined the SOD activity in lactobacilli. In the present paper we show that some strains of lactobacilli contained SOD activity which is much lower than that of *Streptococcus mutans*,[8)] *Bacillus subtilis*,[8)] or *Escherichia coli*.[9)]

Experimental

Organisms and Growth Conditions *L. acidophilus* ATCC 4356, *L. murinus* ATCC 35020, *L. plantarum* ATCC 8014, and *L. fermentum* ATCC 9338 were obtained from the American Type Culture Collection; *L. acidophilus* CRL 358, *L. plantarum* CRL 353, *L. casei* CRL 431, and *L. fermentum* CRL 251 were obtained from the Centro de Referencia para Lactobacilos Culture Collection; *L. buchneri* NCDO 110 was obtained from the National Culture of Dairy Organisms.

The basal media for the growth of these microorganisms (LAPT) were the one described by Raibaud *et al.*,[10)] and Rogosa medium (MRS).[11)] The cells were first precultured in a tube with 5 ml of medium, inoculated into an Erlenmeyer flask containing 50 ml, and finally grown in 500 ml (LAPTg) at 37 °C for 20 h under aerobic conditions. When the cultures had reached the stationary phase, cells were harvested by centrifugation at 8000 *g* for 20 min at 4 °C.

Preparation of Dialyzed Cell-Free Extract The harvested cells were washed with 0.05 M carbonate buffer, pH 10.2, and suspended to 10 ml with the same buffer. Washed cells were ruptured by two passages through a French-X press. Disruption was performed at $2000\,g \times s \times cm^{-2}$ (28000 psi) and the supernatant obtained by centrifugation at 28000 *g* for 30 min at 4 °C (the cell-free extract) was dialyzed for 18 h against 0.05 M carbonate buffer, pH 10.2. The buffer was changed three times during dialysis.

Assay of SOD Activity SOD activity in the dialyzed cell-free extract was assayed in terms of reduction of nitro blue tetrazolium (NBT) by the superoxide radical (O_2^-) generated by the xanthin–xanthine oxidase system.[12)] 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.*.[12)] The extract volumes used in the enzyme reactions were 0.2, 0.5 and 0.8 ml; these gave similar results in the majority of cases except in the strains grown in MRS broth, where enzyme saturation by substrate was seen with 0.5 and 0.8 ml.

Redistilled water was used in order to avoid interference with the enzyme by contaminating metal ions.

Protein Determination The protein content of the samples was determined by the method of Lowry *et al.*,[13)] using bovine serum albumin as a standard.

Results and Discussion

Xanthine is able to transfer electrons to NBT *via* oxygen. This effect is inhibited by SOD or Mn^{2+} ion. In order to avoid misleading results it is necessary to work with dialyzed cell-free extract and the reaction mixture must include ethylenediamine tetraacetic acid (EDTA) as a complexing agent.

In all cases the protein content was higher than 7 mg/ml and the SOD activity was heat-labile, since after heating in a boiling water bath for 30 min the dialyzed cell-free extract lost their SOD activity. SOD activity of the strains studied in this work was resistant to 7.0 μmol of cyanide or azide, with the exception of that of *L. murinus*, which was sensitive. The control consisted of the complete system without cell-free extract; when xanthine, NBT, or xanthine oxidase was omitted, absorbance was negligible.

SOD Activity in Dialyzed Cell-Free Extracts Obtained from Obligated Homofermentative Lactobacilli The results are shown in Table I. The specific SOD activity in *L. acidophilus* ATCC 4356 and *L. acidophilus* CRL 358 increased when the Mn^{2+} ion concentration in the culture media was higher.

The specific SOD activity in *L. murinus* ATCC 35020 changed when the cells were grown in the presence of 3×10^{-3} mM (Table I(a)), 3×10^{-2} mM (Table I(b)) or 3×10^{-1} mM (Table I(c)) Mn^{2+}. Cells grown in LAPTg containing 3×10^{-2} mM Mn^{2+} and those grown in MRS 3×10^{-1} mM Mn^{2+} showed the same SOD activity. The results in part I(a) and (b) of the table were those obtained working with 0.8 ml of extract, while the data in part I(c) were obtained with 0.2 ml. Furthermore, the enzyme ac-

TABLE I. SOD Activity[a] in Dialyzed Cell-Free Extracts Obtained from Homofermentative Lactobacilli

	Inhibition (%)	Proteins (mg/ml)	Specific activity (unit/mg protein)
a) Cell growth in LAPTg + 3×10^{-3} mM Mn^{2+}			
Control	—	—	—
L. acidophilus ATCC 4356	40.00	9.20	0.109
L. acidophilus CRL 358	40.00	8.80	0.114
L. murinus ATCC 35020	25.20	9.65	0.065
L. casei CRL 431	24.90	9.90	0.063
L. plantarum ATCC 8014	63.56	10.60	0.150
L. plantarum CRL 353	20.55	8.30	0.062
b) Cell growth in LAPTg + 3×10^{-2} mM Mn^{2+}			
Control	—	—	—
L. acidophilus ATCC 4356	80.00	9.80	0.204
L. acidophilus CRL 358	74.80	8.40	0.223
L. murinus ATCC 35020	46.00	10.00	0.115
L. casei CRL 431	40.49	8.05	0.126
L. plantarum ATCC 8014	63.56	10.60	0.150
L. plantarum CRL 353	51.42	7.90	0.163
c) Cell growth in MRS[b]			
Control	—	—	—
L. acidophilus ATCC 4356	33.07	8.20	0.403
L. acidophilus CRL 358	34.65	8.05	0.430
L. murinus ATCC 35020	11.02	9.40	0.117
L. casei CRL 431	40.32	7.25	0.139
L. plantarum ATCC 8014	43.15	9.15	0.118
L. plantarum CRL 353	49.60	9.75	0.127

a) These data are the means of 4 experiments. *b*) Only 0.2 ml of dialysate obtained from cells grown in MRS was used since 0.5 and 0.8 ml showed enzyme saturation by substrate.

tivity present in this strain was inhibited by the addition of 2.5 mM potassium cyanide or sodium azide, suggesting the presence of a Cu/Zn SOD. Further studies should be carried out in order to account for this difference, because there is only one prokaryote cell known to contain Cu/Zn enzyme.[14]

SOD Activity in Dialyzed Cell-Free Extracts Obtained from Facultative Homofermentative Lactobacilli The results are shown in Table I. *L. plantarum* ATCC 8014 and *L. plantarum* CRL 353 showed a higher level of SOD activity in LAPTg with 3×10^{-2} mM Mn^{2+} (Table I(b)) than in MRS (Table I(c)), while the opposite was observed for *L. casei* CRL 431. The highest level of SOD activity for this group was 0.163 U/mg protein; the low values of activity found in our experiments, are not necessarily inconsistent with the negative results obtained in previous studies in which certain investigators attempted to demonstrate the existence of SOD activity in *L. plantarum* ATCC 8014,[1,15] bearing in mind that results obtained with different methods are not directly comparable.

SOD Activity in Dialyzed Cell-Free Extracts Obtained from Heterofermentative Lactobacilli The results are shown in Table II. When this group of strains was grown in LAPTg containing 3×10^{-3} mM Mn^{2+} we were unable to detect SOD activity even when working with dialyzed extracts with high protein content (11—12 mg/ml). The cells cultured in MRS (Table II(b)) showed higher SOD activity than those cultured in LAPTg with 3×10^{-2} mM Mn^{2+} (Table II(b)). As far as we know, there are no precedents in the literature for SOD activity in heterofermentative lactobacilli.[6,7]

The data suggest that SOD activity is maximum in

TABLE II. SOD Activity[a] in Dialyzed Cell-Free Extracts Obtained from Heterofermentative Lactobacilli

	Inhibition (%)	Proteins (mg/ml)	Specific activity (unit/mg protein)
a) Cell growth in LAPTg + 3×10^{-2} mM Mn^{2+}			
Control	—	—	—
L. fermentum ATCC 9338	41.30	10.90	0.095
L. buchneri NCDO 110	25.91	10.30	0.063
L. fermentum CRL 251	44.94	10.60	0.106
b) Cell growth in MRS			
Control	—	—	—
L. fermentum ATCC 9338	51.36	10.80	0.119
L. buchneri NCDO 110	35.41	11.50	0.077
L. fermentum CRL 251	50.19	10.10	0.124

a) These data are the means of 4 experiments.

obligate homofermentative strains, medium in facultative strains and minimum in heterofermentative strains. The highest SOD activity was detected in dialyzed cell-free extract obtained from *L. acidophilus* CRL 358 grown in MRS broth (Table I(c)) while the lowest was determined in *L. buchneri* NCDO 110 grown in LAPTg with 2×10^{-2} mM Mn^{2+} (Table II(a)). With the exception of *L. murinus* ATCC 35020, the strains studied seem to possess Mn–SOD since their enzymes were resistant to cyanide and azide ions.[16,17]

Archibald and Fridovich[1] studied O_2^- scavenging activity in undialyzed *L. plantarum* cell extract, which was obtained from cell cultures in MRS broth; this medium contains Mn^{2+} ions, which were not eliminated because the authors worked without dialysis. *L. plantarum* ATCC 8014 possesses an aerobic metabolism involving dinucleotide reduced form (NADH)–nicotinamide adenine dinucleotide phosphate reduced form (NADPH), lactate and pyruvate oxidases. The question of whether or not SOD is present in *L. plantarum* remains open.[2] Götz *et al.*[15] studied the superoxide degrading activity in cell-free extract of the same microorganism, and attributed this activity to non-proteinaceous compounds. In our study we used the same strain of *L. plantarum* as the above-mentioned authors with a SOD activity of 0.150 U/mg protein. A further point to be taken into consideration is that when we worked with obligate homofermentative strains grown in MRS with high specific activity (Table I(c)), we obtained enzyme saturation by the substrate.

Overall, our results suggest that manganese in the growth medium is necessary for the induction of SOD activity and it is possible to demonstrate the presence of SOD in dialyzed cell-free extract obtained from lactobacilli.

Acknowledgments This work was partially supported by grants from Secretaría de Ciencia y Tecnología, República Argentina, 1985—1986, and PID N° 3-09100/85 CONICET.

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