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Characterization of Superoxide Dismutase in *Lactococcus lactis*

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Abstract The superoxide dismutase (SOD) in *Lactococcus lactis* was measured quantitatively and qualitatively under various culture conditions. The *L. lactis* SOD was induced by oxidative stress. As the concentration of paraquat to produce superoxide radicals increased, the growth of *L. lactis* decreased with concomitant increase of SOD activity. The SOD activity was found to be growth-phase dependent: when aerobically grown cells entered to the stationary phase, the activity increased gradually until the late stationary phase. From inhibition studies, *L. lactis* SOD was found to be insensitive to KCN and H_2O_2 which are known to inhibit Cu/ZnSOD and FeSOD, respectively. Moreover, as the concentration of manganese in the medium increased, the activity of SOD also increased. These data strongly suggested that *L. lactis* possessed a single manganese-containing SOD (MnSOD). Finally, a putative *sod* gene fragment of 510 bp was identified in *L. lactis* using a polymerase chain reaction (PCR) with degenerate primers designed from the deduced DNA sequences of known SOD genes.

Key words: *Lactococcus lactis*, superoxide dismutase, metal supplementation, PCR

Lactococcus lactis, a gram-positive and facultatively anaerobic lactic acid bacterium, is widely used in fermented food production as a starter culture. In general, the growth of lactic acid bacteria (LAB) does not necessarily require anaerobic growth conditions. A number of LAB containing NADH oxidase and NADH peroxidase can convert molecular oxygen or hydrogen peroxide into its oxidative form to regenerate NAD^+ [9]. If LAB are particularly cultured in aerobic conditions or sometimes in anaerobic conditions, superoxide (O_2^-) radicals are generated from molecular oxygen by chemical and enzymatic reactions [15]. Superoxide radicals produced by this mechanism cause certain toxicities and some mutations in LAB as well as other living cells involved in oxygen [12]. Therefore, to protect themselves from oxidative stresses such as superoxide radicals, LAB and other organisms have evolved complex oxidative defense systems [11]. Unlike other aerobic organisms, including *Escherichia coli*, some LAB can use either nonenzymatic or enzymatic dismutation systems to remove superoxide radicals. Several aerotolerant LAB, including *Lactobacillus plantarum*, have evolved nonenzymatic dismutation systems which can accumulate high intracellular levels of Mn^{2+} and stoichiometrically remove superoxide radicals [1, 2, 14]. In the enzymatic dismutation systems involved in LAB and other aerotolerant bacteria, SOD dismutates superoxide radicals by catalyzing the enzymatic reaction

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

SODs, one of the most important factors in oxidative defense systems, are metalloenzymes which catalyze dismutation of O_2^- into H_2O_2 and O_2 [19]. There are three types of SODs known (MnSOD, FeSOD, and Cu/ZnSOD) that contain either manganese, iron, or a combination of copper and zinc as their catalytic metal cofactors at the active sites. MnSODs are found in prokaryotes and in the mitochondria of eukaryotes, whereas FeSODs are found in prokaryotes and in the chloroplasts of eukaryotes [25]. Although Cu/ZnSODs are widely present in eukaryotes, they are also present in a small number of gram-negative bacteria. MnSODs and FeSODs, which are structurally closely related to each other, have probably diverged from a common ancestor, whereas Cu/ZnSODs appear to have evolved independently [25].

This paper characterizes SOD activity in *L. lactis*, which appears to be a single MnSOD, through inhibition studies and metal supplementation experiments. Furthermore, a putative *sod* gene amplified by PCR was identified using degenerate primers designed from the sequence data of other *sod* genes [21].

MATERIALS AND METHODS

Bacterial Strains, Media, and Chemicals

The bacterial strains used in this study were *Lactococcus lactis* subsp. *lactis* ATCC 19435 and *Streptococcus mutans*

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ATCC 25175 which were obtained from the Korean Collection for Type Cultures. As positive and negative control strains for SOD activity, *Escherichia coli* HB101 containing plasmid pDTI-5 and *E. coli* QC774, a *sodA sodB* double mutant, were obtained from D. Touati (University de Paris, France) [17]. *L. lactis* subsp. *lactis* and *S. mutans* were grown in M17 broth containing 0.5% glucose (M17G) at 30°C and 37°C, respectively [27]. Anaerobic growth conditions were maintained under CO_2 gas. Aerobically grown cultures were grown with shaking at 200 rpm and without shaking (still culture). The culture/flask ratio was 1/5 for aerobic grown cultures. *E. coli* strains were grown in Luria-Bertani (LB) broth at 37°C with vigorous agitation.

Ampicillin and paraquat purchased from Sigma Chemical Co. (U.S.A.) were used at concentrations of 50 μg/ml and from 1 μM to 10 mM, respectively. $MnSO_4$ and $FeSO_4$ at 0.1 μM to 10 mM were used for metal supplementation experiments in which iron and manganese were supplemented in M17G. KCN and H_2O_2 were used at concentrations of 2 mM and 15 mM, respectively.

Preparation of Cell-Free Extracts (CFEs)

CFEs were prepared as described previously [8]. Briefly, cells were harvested by centrifugation at 10,000 ×g for 5 min at 4°C, washed twice with a 50 mM potassium phosphate buffer containing 0.1 mM EDTA [pH 7.8; KPi-EDTA buffer], and resuspended in the same buffer. The cell suspensions were disrupted by sonication for 30-s bursts for a total of 10 min on ice. Cellular debris was removed by centrifugation at 20,000 ×g for 20 min at 4°C, and the supernatant was used as the CFE.

Enzyme Assays

The protein concentration was determined using the Bradford method [5]. SOD activity was measured by the cytochrome c method, and the specific SOD activity was expressed as McCord/Fridovich units [19]. Relative SOD activity was expressed as a percentage of the inhibition rate (%). To visually identify the SOD activity, the proteins in the CFEs were separated on 12% native polyacrylamide gels and stained for SOD activity with nitroblue tetrazolium (NBT) [4].

Inhibition Experiments

It is known that KCN inhibits Cu/ZnSOD, and H_2O_2 inhibits FeSOD or Cu/ZnSOD [3, 6]. In this study, Cu/ZnSOD and FeSOD inhibition experiments were carried out to identify the type of SOD. In the Cu/ZnSOD inhibition experiment, after CFEs were separated on 12% nondenaturing gel electrophoresis, one of two duplicate gels was incubated with 2 mM KCN and a Kpi-EDTA buffer, whereas the other was incubated with only the Kpi-EDTA buffer, and they were then stained with NBT to ascertain the SOD activity [4, 7]. In the FeSOD inhibition experiment, the same method was used as in the Cu/ZnSOD inhibition experiment except with the use of 15 mM H_2O_2 in place of KCN.

DNA Amplification

Genomic DNA was extracted from the bacterial strains as previously described [16]. DNA amplification was performed in a final volume of 100 μl containing 50 ng of genomic DNA, 0.1 μM of each primer, 200 μM of each dNTP, and 2 units of *Taq* DNA polymerase in a buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$). The PCR mixture was subjected to a denaturation step (3 min at 95°C), followed by 35 cycles of amplification (120 s of annealing at 37°C, 90 s of elongation at 72°C, and 30 s of denaturation at 95°C), and to final annealing (4 min at 37°C) and elongation (12 min at 72°C) steps. The coding-strand primer [*d1*] and complementary-strand primer [*d2*] devised from the sequence data of other *sod* genes from gram-positive bacteria were used as degenerate primers to amplify the *sod* genes from *L. lactis* and *S. mutans* [20]. The sequences of the corresponding oligonucleotides, *d1* and *d2*, were 5'-CCITAYICITAYGA-YGCIYTIGARCC-3' and 5'-ARRTARTAIGCRTGYTCCCAIACRTC-3', respectively.

Results and Discussion

Induction of SOD in *L. lactis* by Oxygen

SOD in *E. coli* and other aerobic microorganisms is known to be induced by oxygen [17, 20]. To determine whether the expression of SOD in *L. lactis* is also affected by oxygen, cultures were grown under anaerobic and aerobic conditions and the specific SOD activities were measured using the cytochrome c method. The specific SOD activities in the CFEs of the anaerobic, aerobic (still), and aerobic (shaking at 200 rpm) cultures were 21±4.6, 105±13.3, and 157±10.5 U/mg, respectively. These data indicate that SOD in *L. lactis* is induced by oxygen as in *E. coli* and other aerobic microorganisms [17, 20]. However, this oxygen effect was not observed in other LAB. *Streptococcus thermophilus*, a commercially important lactic acid bacterium, did not show any increased SOD activity upon aeration [7].

Effects of Paraquat or Growth Phase

In order to substantiate the effect of oxidative stress, cells were exposed to paraquat which produced superoxide radicals in a culture medium [17]. As shown in Fig. 1, as the concentration of paraquat increased, the growth of *L. lactis* decreased, while the activity of SOD increased. This result is in good agreement with previous reports on the effect of oxidative stress on the SOD activity of other

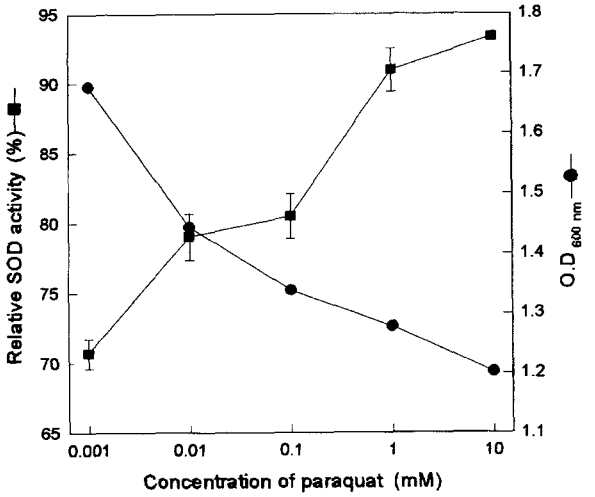

Fig. 1. Effects of paraquat on the relative SOD activity and survival of *L. lactis*. CFEs were prepared from cells after 8 h of incubation upon exposure to paraquat. The cell growth and SOD activity were measured simultaneously. Relative SOD activity was expressed as a percentage of the inhibition rate in reduction of cytochrome c by superoxide radicals (%). Prior to exposure to various concentrations of paraquat, a fresh M17G medium was inoculated with a overnight culture of *L. lactis* at the dilution ratio of 1:20.
Symbols: relative SOD activity (■), optical density at 600 nm (●).

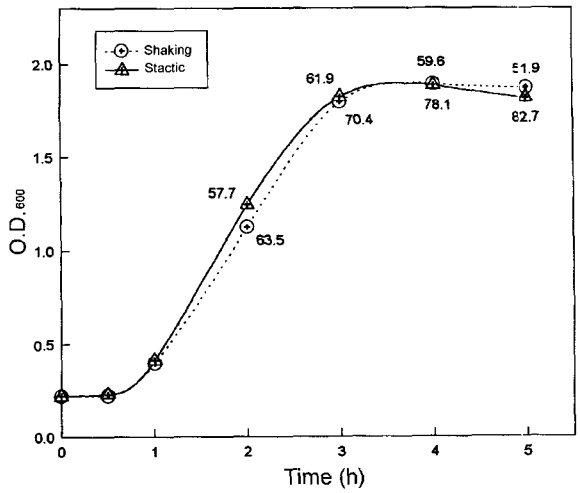

Fig. 2. Effect of growth phase on the aerobic expression of SOD in *L. lactis*. Cultures were aerobically grown with shaking (dashed line) and without shaking (solid line) to the indicated growth phase which represents the optical density at 600 nm ($O.D._{600}$). The numbers over the lines indicate the relative SOD activity (%) in the cultures grown without shaking at the indicated optical density, while the numbers under the lines indicate the activity in the cultures grown with shaking. Relative SOD activity was expressed as a percentage of the inhibition rate in reduction of cytochrome c by superoxide radicals (%).
Symbols: with shaking at 200 rpm (○), without shaking (still) (△).

bacteria [13, 17]. Accordingly, this study confirmed that SOD in *L. lactis* was also induced by oxidative stress such as paraquat. This was in contrast to the studies on *S. thermophilus* which showed that SOD activity was not induced by paraquat [7].

SODs are induced in a number of bacteria during the stationary phase [24, 26], however, little is known about the relationship between the expression of SOD and the growth phase in *L. lactis*. To determine if SOD activity was affected by the growth phase of *L. lactis*, the expression of SOD was examined throughout the growth cycle. Cultures were grown aerobically with and without shaking. The relative SOD activities were measured at hourly intervals during the period of the middle of the log phase until the death phase. The relative SOD activities were found to increase upon entry into the stationary phase in the cultures grown without shaking, while the activities in the cultures grown with shaking increased until the death phase (Fig. 2). These results indicate that SOD in *L. lactis* is expressed in a growth-phase-dependent manner. It is suggested that the expression of SOD in *L. lactis* is affected by both oxidative stress and other stresses, presumably starvations, toxic reagents, and acids [23].

L. lactis Contains MnSOD

SODs are known as metalloenzymes. According to their metal cofactors, three types of SODs (MnSOD, FeSOD, Cu/ZnSOD) have been identified. MnSODs and FeSODs are structurally closely related to each other, whereas Cu/ZnSODs are structurally and evolutionally distinct [25]. In this study, several inhibition experiments were performed to determine the type of SOD in *L. lactis*. CFEs from aerobically grown *L. lactis* cultures were prepared and assayed for qualitative SOD activities. Staining for SOD activity revealed a band that was not inhibited by KCN, which is known to inhibit Cu/ZnSOD, therefore, the possibility of Cu/ZnSOD was ruled out (data not shown). However, the presence of H_2O_2, which inhibited the FeSOD in the NBT staining solution, resulted in a partial inhibition of the SOD activity (data not shown). This result suggested that *L. lactis* possessed some FeSOD, however, it was not clear whether the SOD activity band was indeed inhibited by H_2O_2, because such partial inhibition was also observed by other researchers working on *S. thermophilus* [7]. Accordingly, a modified experiment was performed in

Fig. 3. Effect of H_2O_2 on activity of SOD in CFEs of *L. lactis*. CFEs (80 μg of protein/lane) were prepared and fractionated through native PAGE. Gels were treated with catalase (50 U/ml) during the staining activity. Gel A, No treatment; Gel B, treated with 15 mM H_2O_2 for 30 min.

which catalase was used to remove any residual H_2O_2 during the staining activity, because the residual H_2O_2 that did not take part in the inhibition reaction might have produced an unknown effect. As shown in Fig. 3, no inhibition was observed in this modified experiment. Therefore, it was concluded that *L. lactis* did not possess any FeSOD. As a result, it is highly likely that *L. lactis* possesses a single MnSOD, but not Cu/ZnSOD nor FeSOD.

Effects of Metal Ions

In order to corroborate the findings, metal supplementation experiments were performed. A medium was supplemented with increasing concentrations of either manganese or iron, and the relative SOD activities were measured. In the iron supplementation experiment, an M17G medium was supplemented with $FeSO_4$ at concentrations ranging from 0.1 μM to 10 mM. As shown in Fig. 4A, the relative SOD activity in *L. lactis* did not increase with an increasing concentration of $FeSO_4$. This result suggested that *L. lactis* did not possess FeSOD. In other organisms containing FeSOD (e.g. *E. coli*), SODs were induced by an iron supplement [10, 22]. Interestingly, at a high concentration (10 mM) of $FeSO_4$, *L. lactis* SOD was inhibited (Fig. 4A).

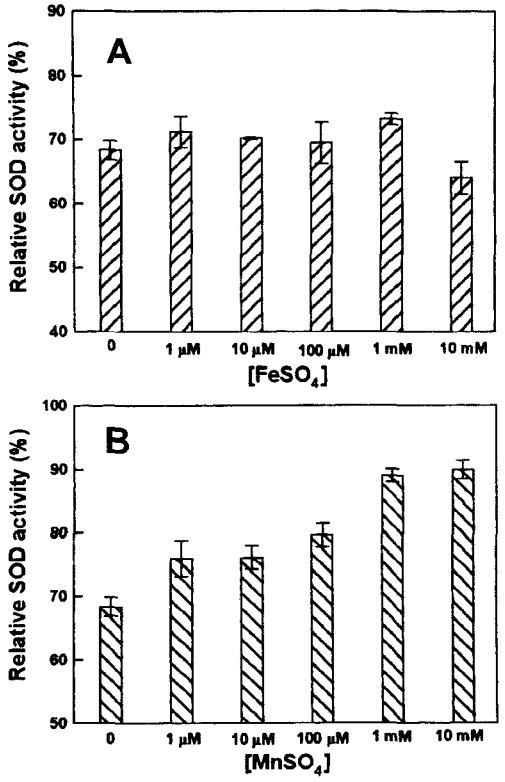

Fig. 4. Effects of iron and manganese on the relative SOD activity in *L. lactis*. $FeSO_4$ (A) or $MnSO_4$ (B) was added to an M17G medium at concentrations ranging from 1 μM to 10 mM, respectively. Cells were harvested at the stationary phase of the growth cycle and CFEs were extracted. CFEs prepared from *L. lactis* were assayed for the relative SOD activities and proteins.

Manganese supplementation was also examined in the same way as described above for the iron supplementation experiment. When the concentration of $MnSO_4$ increased from 0.1 μM to 10 mM, the relative SOD activity gradually increased with an increasing concentration of $MnSO_4$ (Fig. 4B). This result indicated that the *L. lactis* SOD induced by manganese could use manganese as the metal cofactor, but not iron or zinc. These results are in line with previous findings related to the MnSODs of other bacteria [18, 22].

Detection of MnSOD by PCR Using Degenerate Primers

In order to obtain additional genetic evidence for the presence of a single MnSOD in *L. lactis,* a PCR amplification was performed on the genomic DNA of *L. lactis* using degenerate primers designed from the sequence data of other *sod* genes [21]. The DNA of *S. mutans* was used as a positive control which yielded a 480 bp PCR product. The PCR products were separated using agarose gel electrophoresis and a fragment of about 510 bp was observed in *L. lactis* (Fig. 5). Although the sizes of the PCR products were different between the two organisms, it was suggested that the PCR product was probably a putative MnSOD gene fragment of *L. lactis*. Further studies to determine whether the fragment was indeed a MnSOD gene, are underway. The PCR fragment from *L. lactis* is currently being cloned to determine the DNA sequence and deduced amino acid sequence. This result will conclusively answer the question

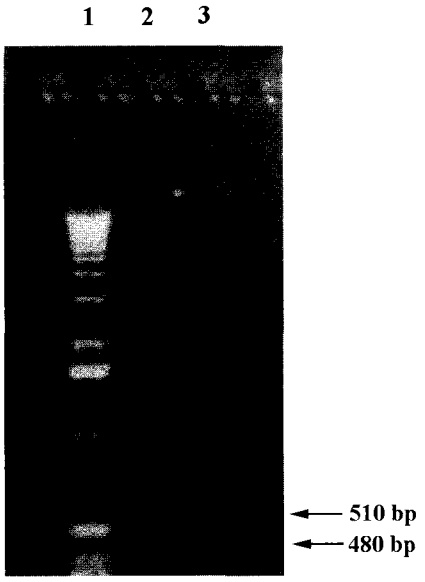

Fig. 5. Agarose gel electrophoresis of putative MnSOD gene fragment amplified by PCR on genomic DNA of *L. lactis* using degenerate primers.

The genomic DNA of *S. mutans* was used as a positive control for *sod* gene amplification. Electrophoresis was carried out on a 1% agarose gel. Lane 1, 1 kb ladder (marker); Lane 2, *S. mutans*; Lane 3, *L. lactis*.

of whether the SOD in *L. lactis* is indeed MnSOD, and open a research avenue to study the physiological role and regulation of MnSOD in this organism [13, 20].

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