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Study on the Intracellular Superoxide Dismutase Produced by *Bacillus circulans*

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*Bacillus circulans*가 생산하는 Superoxide Dismutase에 관한 연구

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Distribution of superoxide dismutase (SOD) which catalyzes the dismutation of superoxide radicals to hydrogen peroxide and oxygen has been examined in various genera of bacteria. SOD was produced by various bacteria independent of genus and species with variation in superoxide dismutase activity of each bacteria. *Bacillus circulans* which produced relatively large amount of SOD was selected and used to investigate the optimum culture conditions and further studies. The compositions of optimum culture medium for the enzyme production were 1% glucose, 2% polypeptone, 0.1% NaCl, and 0.2mM of methyl viologen and initial pH was 6.0. The highest enzyme production was observed after 20 hours of cultivation at 30°C on a reciprocal shaker. The enzyme activity was maintained stably for a relatively long period by the addition of 5% ethanol in pH 5.0, 0.01M acetate buffer.

The superoxide radicals, the first toxic products formed by the univalent reduction of oxygens, are produced during the various biological reaction in mitochondrial electron transport system (1), xanthine oxidase system (2), leucocyte and macrophage (3). The defense against the superoxide in biological system is achieved specifically and efficiently by the enzyme superoxide dismutase which scavenges the superoxide-free radical, by way of the dismutation reaction: $O_2^- + O^-_2 + 2H^+ \rightarrow H_2O_2 + O_2$ (4). This protective enzyme has been found to be ubiquitous in oxygen-metabolizing organisms, but absent from obligate anaerobes. Owing to its important role in the defensive machanism (1-5), SOD can be applied in practical uses for not only medical treatment in the diseases caused by O^-_2, such as troubles of liver (6), skin (7), eye (8), brain (9), radiation sickness (10), lipid peroxidation (11), inflammation (12), rheumatism (13), self-immune disease (14), and arteriosclerosis (15) but also protecting normal cells from damages caused by anti-cancer medicine by using combined treatment with SOD (16). Also several reports on the relationship between age-related modification and physiological function of SOD in tissues were made (17).

The enzyme SOD is a metalloprotein having a redox metal at the active site and three distinct types of SOD have been isolated (18). These are the copper-zinc, manganese, and iron SOD. The copper-zinc SOD, the most studied form, is characteristic of the cytosols of eukaryotic cells, fungi (19) and mammalian cells (4). Two kinds of manganese SOD were isolated, one of which is associated with the mitochondria of eukaryotes (4,18,20) and the

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other is found in the matrix or periplasmic space of bacteria (21,22). The iron SOD has been found in bacteria (23) and blue-green algae (24) and it is synthesized in anaerobically as well as in aerobically grown cells and is therefore considered to be constitutive (5). However its function has not been well understood. The iron and manganese enzymes are distinct from copper-zinc enzyme in the point of amino acid homology (25) and cyanide-insensitivity (21,22).

Superoxide dismutase has been isolated and characterized from a variety of respiring eukaryotic organisms, especially mammalian cells such as human brain and erythrocyte, heart and liver of rat, cow, and horse. However, little has been reported on SOD from aerobic bacteria except for in the cases of *Streptococcus mutans* (21) and *Escherichia coli* (22,23).

In this paper, the distribution of superoxide dismutase which plays an important role of defenses in biological system has been examined in various bacteria especially in aerobes. Furthermore, the isolation of SOD which seems very valuable as clinical medicine was carried out from aerobic bacteria, *Bacillus circulans* IFO 3329 and investigation on media composition, other culture conditions and regulation of biosynthesis of SOD was also performed for the purpose of clinical application and establishment of mass production system.

Materials and Methods

Strain and cultivation

Several stocked strains were grown in test tubes (22×200 mm) containing 8 m*l* of basal medium (1% glucose, 1% polypeptone, 1% meat extract 0.1% NaCl, and adjusted to pH6.0) under aeration (120 stroke/min) at 30°C for 72hr. Crude enzyme solution prepared from each bacteria was used to measure SOD activity.

In order to investigate the effects of carbon and nitrogen sources, inorganic salts, initial pH and methyl viologen on SOD production, the selected strain, *Bacillus circulans* IFO 3329, was grown at 30°C for 24hr in 500 m*l* shake flask containing 100 m*l* of each indicated media composition with addition of 3 m*l* of seed culture.

Crude enzyme preparation

Cultured cells were harvested by centrifugation at 10,000×g for 10 min, washed twice with 0.85% NaCl solution, resuspended in 0.05M phosphate buffer (pH 7.0) and then disrupted by sonic oscillation for 3 min with 1 min pause for cooling. The cell debris was removed by centrifugation at 13,000×g for 20 min and then the resulting cell-free extracts were used as crude enzyme solution to assay SOD activity.

Assay of enzyme activity

SOD activity was conveniently assayed in terms of its ability to inhibit the autoxidation of epinephrine to adrenochrome, which exhibits an absorption maximum at 480 nm (26). Enzyme assay was performed at 20°C by mixing epinephrine stock solution (pH 2.0) 0.3 m*l*, sodium carbonate buffer (pH 10.2) 3 m*l* containing EDTA, and adequately diluted enzyme solution 0.3 m*l* to a final concentration of 3×10^{-4}M epinephrine, 3×10^{-4}M EDTA and 0.05 M carbonate buffer. The reaction was accomplished in cuvette for 3 min with recording the absorbances of the reaction at 480 nm by time-scanning. One unit of SOD was defined as that amount which caused 10% inhibition of blank autoxidation.

Measurement of optical density and determination of cell mass

Bacterial concentration was measured by absorbance of culture at 660 nm and all the spectrophotometric assays were performed with Shimadzu UV-240.

Results and Discussion

Selection of SOD-producing bacteria

Distribution of SOD in various species of bacteria was examined. SOD was produced by various bacteria independent of genus and species with variation in superoxide dismutase activity of each bacteria as shown in Table 1. Some reasons might be presumed as follows for the result that the

Table 1. Distribution of superoxide dismutase in various species of bacteria

Strains	Growth (O.D. at 660 nm)	SOD activity (units/mg cell)
Aerobacter aerogenes IFO 3317	1.7	1.3
Bacillus circulans IFO 3329	5.0	1.2
Achromobacter acerius IFO 3166	1.7	1.1
Agrobacterium tumefacience IAM 1037	6.7	0.8
Bacillus sphaericus IFO 3528	2.5	0
Brevibacterium ammoniagenes IFO 1207	5.8	0
Micrococcus luteus IFO 3763	7.0	0.3
Pseudomonas polycolor IFO 3918	4.9	0
Proteus vulgaris IFO 3851	3.8	0.8
Sarcina lutea IFO 3232	7.3	0.2
Staphylococcus aureus IFO 3060	1.2	0
Xanthomonas campestris IAM 167	3.1	1.0

four species of bacteria never produced SOD at all even though they were all aerobes and considered to have both Mn-SOD and Fe-SOD. Either too small amount of SOD, or unsuitable media and culture conditions for the enzyme production, or inactivation during preparation of crude enzyme solution might cause difficulty in detection of SOD activity. *Bacillus circulans,* which is Gram-positive and aerobic bacteria, was selected for the further studies in consideration of cell lysis either by sonication or lysozyme, facile application to fermentation industry and safe organism from a biohazard point of view.

Culture conditions for SOD formation

B. circulans IFO 3329 was grown in the basal medium with a variety of carbon sources. Among the carbon compounds tested in this work, glucose and maltose were appeared to be most effective for SOD synthesis but galactose and ribose were appeared to be uneffective (Table 2). On the contrary, it was reported by Hassan *et al* (27) that in *E. coli* which is a facultative anaerobe, synthesis of SOD was decreased in the presence of ample glucose. It

Table 2. Effect of carbon sources on the production of SOD

Carbon sources	Final pH	Growth (O.D.at 660 nm)	SOD activity (units/mg cell)
None	8.0	3.39	0.7
Glucose	7.5	5.16	1.4
Arabinose	8.0	6.08	0.9
Galactose	7.5	5.87	0.3
Mannose	7.5	4.53	1.0
Mannitol	8.0	7.24	0.7
Sorbitol	7.5	4.78	0.8
Fructose	7.5	3.48	0.9
Inositol	8.0	4.03	0.8
Lactose	8.5	4.32	1.1
Sodium acetate	9.0	2.87	1.1
Sodium citrate	9.0	1.00	0.9
Xylose	8.0	4.40	0.9
Sorbose	8.5	1.05	0.9
Maltose	8.5	3.97	1.4
Raffinose	7.8	4.37	1.1
Trehalose	7.2	7.45	1.0
Glycerin	6.8	10.29	1.2
Ribose	7.4	4.25	0.6

was further demonstrated that this effect of glucose was not due to catabolite repression but due to a diminished rate of production of O^-_2 in the presence of ample glucose. However in this work, the production of SOD in *B. circulans* was not decreased by 1% glucose but increased slightly. That is probably because this microbe is not a facultative anaerobe but an obligate aerobe and therefore always needs nearly constant oxygenation and resultingly constant production of O^-_2 which is actual inducer of SOD. It is also supposed that 1% glucose is not enough to diminish the rate of O^-_2 production.

The effect of nitrogen sources on the formation of SOD was also determined. Several sources of organic and inorganic nitrogen compounds were added to the basal medium instead of 1% meat extract and 1% polypeptone. As shown in Table 3, 2% polypeptone was favorable for the formation of SOD and the amount of SOD obtained from or-

Table 3. Effect of nitrogen sources on the production of SOD

Nitrogen sources		Final pH	Growth (O.D. at 660 nm)	SOD activity (units/mg cell)
Polypeptone	1%	7.5	5.16	1.4
Meat extract	1%			
Polypeptone	1%	7.5	5.04	1.7
Meat extract	0.5%			
Polypeptone	1%	7.5	3.50	1.2
Polypeptone	2%	7.9	3.75	2.2
Meat extract	0.5%	7.0	2.95	1.4
Meat extract	1%	6.8	3.12	1.0
Yeast extract	1%	5.9	4.15	1.8
Polypeptone	1%			
Yeast extract	0.5%	6.8	6.40	1.6
Meat extract	0.5%			
$(NH_4)_2SO_4$	0.5%	6.1	0.12	0.3
NH_4Cl	0.5%	6.3	0.30	0.2
$NaNO_3$	0.5%	6.2	0.53	0.2
Lysine	1%	6.2	0.39	0.2
Cysteine	1%	5.8	0.17	0.1
Arginine	1%	5.8	0.77	0.3

ganic nitrogen compound was larger than that from inorganic compound. Both inorganic nitrogen compounds and amino acids were unsuitable for the SOD production and cell growth.

The effect of several inorganic salts on the enzyme production was examined with varying their concentrations. Each inorganic salt was added to the medium containing 1% glucose and 2% polypeptone. Table 4 shows that 0.1% of KH_2PO_4, $MnSO_4$ and NaCl were found to be similar effect. However in the case of $FeSO_4$, the assay of SOD activity was impossible because this crude enzyme contained $FeSO_4$ from broth and strikingly accelerated the autoxidation of ephinephrine.

The initial pH of medium composed of 1% glucose, 2% polypeptone, and 0.1% NaCl was adjusted to indicated pH. As shown in Table 5, the initial pH had little effect on growth rate but the enzyme formation was the highest at pH6.0.

Effect of aeration

SOD catalytically scavenges O^-_2 and in so doing

Table 4. Effect of inorganic salts on the production of superoxide dismutase

Inorganic salts		Growth (O.D. at 660 nm)	SOD activity (units/mg cell)
	0.2 %	14.1	1.2
$MgSO_4 \cdot 7H_2O$	0.1 %	11.3	1.1
	0.05%	11.1	1.1
	0.2 %	8.4	1.1
KH_2PO_4	0.1 %	8.2	1.4
	0.05%	4.4	1.1
	0.2 %	3.5	0.5
$MnSO_4 \cdot H_2O$	0.1 %	5.5	1.4
	0.05%	5.9	1.4
	0.2 %	7.5	1.2
$CaCl_2 \cdot 2H_2O$	0.1 %	7.9	1.0
	0.05%	4.5	0.5
	0.2 %	5.7	0.7
$MgCl_2 \cdot 6H_2O$	0.1 %	6.1	0.7
	0.05%	7.0	0.7
	0.2 %	3.9	0.6
K_2HPO_4	0.1 %	4.4	1.1
	0.05%	5.4	0.2
	0.2 %	4.6	0.4
NaCl	0.1 %	6.0	1.4
	0.05%	6.2	0.2
	0.2 %	10.5	0.4
NH_4Cl	0.1 %	6.9	0.6
	0.05%	6.8	0.3
	0.2 %	8.6	0.2
KCl	0.1 %	6.6	0.9
	0.05%	6.5	0.7
	0.2 %	1.1	—
$FeSO_4 \cdot 7H_2O$	0.1 %	4.6	—
	0.05%	9.3	—

-: not determined

Table 5. Effect of initial pH of medium on the production of superoxide dismutase

Initial pH	Final pH	Growth (O.D. 660 nm)	SOD activity (units/mg cells)
5	6.4	4.0	1.2
6	6.7	4.5	2.6
7	6.5	4.7	1.4
8	4.6	4.6	1.4
9	6.7	4.8	1.2

Table 6. Effect of aeration on the production of SOD

Volume of medium (m*l*)	Growth (O.D. at 660 nm)	SOD activity (units/mg cell)
50	5.1	2.7
75	6.0	2.6
100	4.5	2.7
120	5.0	2.6
150	5.1	2.6

provides an important defense against oxygen toxicity. Since, in biological systems, O^-_2 can only be made in the presence of molecular oxygen and since oxygen supply is a variable for many cell types, one might expect that the level of SOD would be responsive to the degree of aeration of culture. It is supposed that decreasing the medium volume per 500 m*l* shake flask would be increase the rate of aeration of culture at constant stroke (120 stroke/min). Therefore *B. circulans* was grown in 500 m*l* shake flask, each containing the indicated volume of medium. As shown in Table 6, the volume of medium had little effect on the SOD production and cell growth. The results obtained here indicate that such aeration with varying media volume was not enongh to overproduce SOD.

Induction of SOD by methyl viologen

The effect of paraquat, a compound which produces superoxide radicals (27), was examined. Methyl viologen (1,1-dimethyl-4'-bipyridinium dichloride) was added to the liquid media by dilution from sterile stock solutions, with varying concentration. As shown in Table 7, 0.2 mM of paraquat increased SOD formation about 2-fold, in comparison to no addition. However the concentration of paraquat, which is effective in inducing SOD, exerted rather lower growth rate and at more than 0.5 mM SOD formation was remarkably decreased.

Table 7. Effect of methyl viologen concentration on the production of superoxide dismutase

Methyl viologen concentration(mM)	Growth (O.D. at 660 nm)	SOD activity (units/mg cells)
0.0	4.5	2.6
0.1	3.5	1.4
0.2	3.7	4.5
0.5	5.6	2.6
0.8	5.3	1.7
1.0	5.3	1.7
1.5	3.0	0.9
2.0	0.3	0

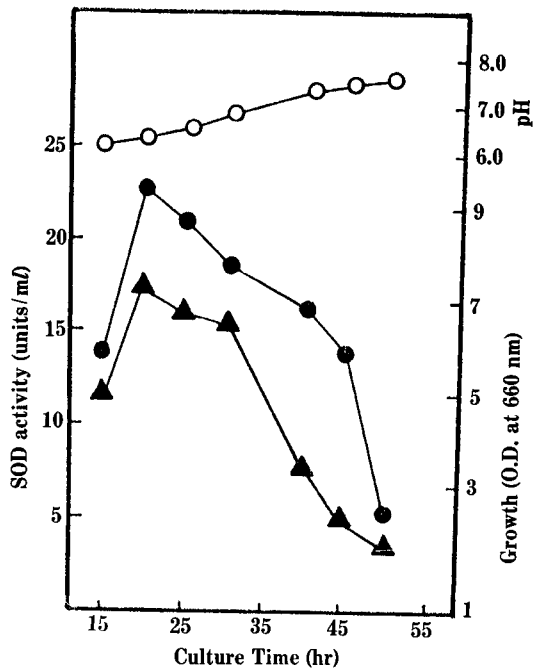

Fig. 1. Time course of growth, pH and SOD activity
-○-: pH -●-: SOD activity -▲-: Growth at 660 nm

Generally paraquat is known to increase intracelluar production of O^-_2 in *E. coli* as well as animal and plant system. In *B. circulans* as well, paraquat probably increases intracellular production of O^-_2 and thus induces the synthesis of Mn-SOD.

Time course of the enzyme production

B. circulans was inoculated in a shake flask containing 100m*l* of optimum medium. Fig. 1 shows the growth of cells, SOD production and the changes of broth pH during cultivation. The maximum production of SOD was obtained after 20 hr incubation. The pattern of cell growth showed similarity to that of enzyme production. The proper condi-

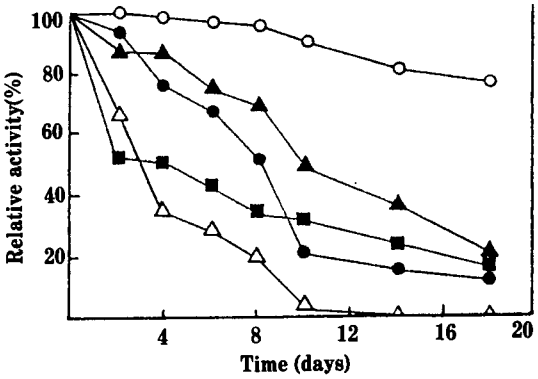

Fig. 2. Effect of some substances on the stabilization of superoxide dismutase
-○-: 5% EtOH -▲-: 10% Glycerin -●-: 1.0 mM $CuSO_4$ -△-: 1.0 mM $ZnCl_2$ -■-: None

tions for the enzyme production were summarized in Table 8.

Table 8. The proper conditions for the production of superoxide dismutase from *Bacillus circulans*

Media	Glucose	1.0%
	Polypeptone	2.0%
	NaCl	0.1%
	Methyl viologen	0.2mM
Conditions	Initial pH	7.0
	Temperature	30°C
	Cultivation time	20hr
	Agitation	120 stroke/min

100 m*l* of media per 500 m*l* shake flask

Stabilization of crude enzyme

B. circulans SOD was very unstable under the conditions as mentioned above. This made it essential that examination on the SOD stabilization be performed. Therefore some kinds of metals thought as cofactors of the enzyme, ethanol and glycerin were tested as stabilizer. As shown in Fig. 2, five percentage of ethanol was found to be very effective for enzyme stabilization whereas the others showed little or adverse effect. Ethanol was selected as a stabilizer and then the effect of ethanol concentrations on SOD stabilization was investigated (Table 9). The stabilizing effect of more than 5% ethanol was alike to that of 5% ethanol. Other investigators (28) examined the effect of organic solvent such as glycerol on protein stabilization on the basis of thermodynamics and kinetics. Sakai *et al.* (29) also reported on an enzyme stabilization by addition of ethanol. However the mechanism of enzyme stabilization by organic solvents was not well understood.

Table 9. Effect of varying ethyl alcohol concentrations on the superoxide dismutase stabilization

Days	SOD activity					
	None	2%	4%	5%	6%	8%
0	21.0	21.0	21.0	21.0	21.0	21.0
3	13.7	18.0	21.0	21.0	21.0	21.0
6	7.3	14.0	21.7	20.0	21.0	20.0
9	3.0	5.7	16.3	17.7	20.0	16.0
14	0.0	0.0	12.7	14.7	13.7	13.3
18	0.0	0.0	12.0	14.0	12.7	13.2

pH stability of crude enzyme

The pH dependence on the stability of the crude enzyme was measured. The enzyme was relatively stable around pH 4.5-7.0 and it was the most stable at pH 5.0. Therefore all the buffer for further analysis was the pH 5.0, 0.01 M acetate buffer containing 5% ethanol.

Purification and some properties of the enzyme SOD from *Bacillus circulans* will be reported elsewhere.

요 약

생체내에서 O_2^-를 특이적으로 제거시켜 줌으로써 의학적 응용가치가 매우 높은 것으로 알려진 superoxide dismutase(SOD)에 대하여 연구하였다. 먼저 이 효소의 활성을 여러 속의 세균을 대상으로 검토해본 결과 비교적 높은 활성을 나타낸 *Bacillus circulans*를 본 실험의 공시균주로 선택한 다음 SOD의 최적 생산 조건을 설정하였다.

SOD의 생산은 각 세균에 따라서 양적인 차이는 있으나 종 및 속에 관계없이 다양하게 존재하는 것으로 나타났다. 효소의 생산 조건은 탄소원으로서 1% glucose, 질소원으로서 2% polypeptone, 무기염으로서 0.1% NaCl을 첨가하였을 때 가장 양호하

였고 최적 pH는 6.0이었다. 이상의 조성을 가진 배지를 500 m*l*용 진탕 플라스크에 100 m*l*를 넣어 30℃에서 20시간 호기적 배양을 하였을때 효소 생산 및 균체량이 최대에 도달하였다. 조효소액은 극히 불안정하여 안정제의 검토가 불가피하였다. 안정제로서는 최종농도가 5% 되도록 ethyl alcohol을 첨가한 경우가 효과적이었으며 pH 안정성은 pH 5.0인 acetate buffer에서 가장 안정하였다. 이상의 조건하에서 조효소액은 20일 이상 동안 약 80%의 잔존활성을 가지고 비교적 안정한 상태로 유지되었다.

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