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# Superoxide Dismutases Exhibit Oxidase Activity on Aldehyde Alcohols Similar to Alcohol Oxidase from *Paenibacillus* sp. AIU 311

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**The relations between oxidase activity on aldehyde alcohols and superoxide dismutase (SOD) were investigated, since the amino terminal amino acid sequence of alcohol oxidase (AOD) from *Paenibacillus* sp. AIU 311, which was specific to aldehyde alcohols, exhibited high similarity to those of SODs containing manganese ($Mn^{2+}$-SOD). *Paenibacillus* AOD had high SOD activity. The SODs containing manganese, iron, or copper and zinc also exhibited oxidase activities on aldehyde alcohols, and the relative values of oxidase activities on aldehyde alcohols to SOD activity of $Mn^{2+}$-SOD were closer to those of *Paenibacillus* AOD compared with those of the other SODs. Thus, SODs had AOD activity on aldehyde alcohols as another enzyme activity, and the *Paenibacillus* AOD and $Mn^{2+}$-SOD were classified into a similar group.**

[**Key words:** glycolaldehyde, glyceraldehyde, alcohol oxidase, superoxide dismutase, *Paenibacillus* sp.]

In our studies of the oxidation of ethylene glycol and its related compounds, we have revealed that a newly isolated strain, *Paenibacillus* sp. AIU 311, produced a new alcohol oxidase (AOD) with high activity on glycolaldehyde and glyceraldehyde, but not on methanol, ethylene glycol or glycerol (1). The $NH_2$-terminal amino acid sequence of this AOD exhibited no similarity to those of AODs from methylotrophic yeasts such as *Candida* and *Pichia*, which catalyze the oxidation of primary alcohols to the respective aldehydes (2–5). We had therefore concluded in our previous report that *Paenibacillus* AOD is a novel enzyme with high specificity to a hydroxy group of aldehyde alcohols (1). In further studies of *Paenibacillus* AOD, we obtained new findings that the $NH_2$-terminal amino acid sequence of this AOD was homologous to that of superoxide dismutase (SOD) containing manganese as cofactor. We therefore analyzed the SOD activity of the *Paenibacillus* AOD. The structures of the SODs have so far been studied in detail (6–9), but other enzyme activities of the SODs have not been studied. We also investigated oxidase activity of SODs on aldehyde alcohols using different three SODs containing manganese, iron, or copper and zinc as cofactors to reveal the relation between SODs and AOD. The present paper describes SOD activity of the *Paenibacillus* AOD and oxidase activity of SODs on aldehyde alcohols such as glycolaldehyde, glyceraldehyde, aldotetrose and aldopentose. The oxidase activities of SODs on short-chain aliphatic alcohols and short-chain aliphatic aldehydes were also analyzed.

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## MATERIALS AND METHODS

**Chemicals** Glycolaldehyde and glyceraldehyde were purchased from Wako Pure Chemical Industries (Osaka). Xanthine oxidase from buttermilk, $Mn^{2+}$-SOD from *Escherichia coli*, $Cu^{2+}Zn^{2+}$-SOD from human erythrocytes, $Fe^{2+}$-SOD from *E. coli*, D-threose, L-threose and D-erythrose were from Sigma Japan (Tokyo). D-Xylose and D-arabinose were from Aldrich Japan (Tokyo). D-Ribose and D-lyxose were from Nacalai Tesque (Kyoto). Horseradish peroxidase (EC 1.11.1.7) was the gift of Amano Enzyme (Nagoya). All other chemicals used were of analytical grade and commercially available.

**Preparation of AOD** *Paenibacillus* sp. AIU 311 was incubated in an ethylene glycol medium consisting of 5% ethylene glycol, 0.1% $NaH_2PO_4$, 0.1% $K_2HPO_4$, 0.2% $NH_4NO_3$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$ and 0.1% yeast extract, pH 5.5, at 30°C for 2 d, and the AOD was purified according to our methods described in our previous paper (1). An enzyme solution with the specific activity of 0.81 unit per mg of protein was used in this study.

**AOD activity** AOD activity was assayed by measuring the rate of hydrogen peroxide formation at 30°C according to our previous report (1) using Spectrophotometer UV-2450 (Shimadzu, Kyoto). The standard reaction mixture contained 50 μmol substrate, 0.24 μmol of 4-aminoantipyrine, 0.78 μmol of *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline sodium salt dihydrate, 2.7 units of

| Strain | 10 | 20 | References |
|---|---|---|---|
| *Paenibacillus* sp. | A F Q L P P L P Y P N D A L E P H I D A Q T M E I H H D R | | 1 |
| *T. thermophilus* | P Y P F K L P D L G Y P Y E A L E P H I D A K T M E I H H Q K | | 11 |
| *E. coli* | S Y T L P S L P Y A Y D A L E P H F D K Q T M E I H H T K | | 12 |
| *B. halodenitrificans* | A K F E L P E L P Y A Y D A L E P T I D K E T M N I H H T K | | 13 |
| *D. radiodurans* | A L A Y T L P Q L P Y A Y D A L E P H I D A R T M E I H H T K | | 14 |

FIG. 1. Alignment of amino terminal amino acid sequence of *Paenibacillus* AOD and $Mn^{2+}$-SOD. Amino terminal amino acid sequence of AOD from *Paenibacillus* sp. AIU 311 was aligned to those of $Mn^{2+}$-SODs obtained by BLAST search. Identical amino acid residues are boxed.

peroxidase, 0.1 mmol of potassium phosphate, pH 6.5, and an appropriate amount of enzyme, in a final volume of 1.0 ml. The reaction was started by addition of enzyme. The formation of hydrogen peroxide was spectrophotometrically followed at 555 nm for 3 min, and the absorbance change per min was obtained ($\Delta A_{e\,555}$). The reaction without enzyme was also carried out under the same conditions, and the absorbance change per min was obtained ($\Delta A_{b\,555}$). The enzyme activity was calculated using the value obtained by subtracting the value of 555 nm without enzyme ($\Delta A_{b\,555}$) from that with enzyme ($\Delta A_{e\,555}$), and a molar absorption coefficient of 16,500 $M^{-1}$ $cm^{-1}$ for the dye formed. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of one micromole of hydrogen peroxide per min under the above conditions.

**SOD activity** Since SOD activity can be assayed using superoxide generated from xanthine by xanthine oxidase, we assayed it by the following two methods.

*Method 1* SOD activity was assayed by measuring the reduction rate of oxidized cytochrome *c* using a slightly modified method of McCord and Fridovich (10) as follows. The reaction mixture containing 50 nmol of xanthine, 10 nmol of oxidized cytochrome *c*, 100 nmol of EDTA, 75 μmol of potassium phosphate buffer, pH 7.8, together with an adequate amount of enzyme in 0.9 ml, was incubated at 25°C for 5 min. Then, 2.7 m units of xanthine oxidase (0.1 ml) was added to the reaction mixture. The formation of the reduced cytochrome *c* was spectrophotometrically measured at 550 nm for 3 min.

*Method 2* SOD activity was assayed by measuring the rate of hydrogen peroxide formation as follows. The reaction mixture containing 50 nmol of xanthine, 100 nmol of EDTA, 75 μmol of potassium phosphate buffer, pH 7.8, color reagent consisting of 0.24 μmol of 4-aminoantipyrine, 0.78 μmol of *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline sodium salt dihydrate and 2.7 units of peroxidase, together with an adequate amount of AOD or SOD in 0.9 ml, was incubated at 25°C for 5 min. The reaction was started by addition of 2.7 m units of xanthine oxidase (0.1 ml). The formation of hydrogen peroxide was spectrophotometrically followed at 555 nm for 3 min, and the absorbance change per min was obtained ($\Delta A_{e\,555}$). The reaction without AOD or SOD was also carried out under the same conditions, and the absorbance change per min was obtained ($\Delta A_{b\,555}$). The enzyme activity was calculated using the value obtained by subtracting the value of 555 nm without enzyme ($\Delta A_{b\,555}$) from that with enzyme ($\Delta A_{e\,555}$), and a molar absorption coefficient of 16,500 $M^{-1}$ $cm^{-1}$ for the dye formed. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of one micromole of hydrogen peroxide per min.

## RESULTS

### Alignment of $NH_2$-terminal amino acid sequence

Using the sequence of 29 amino acid residues from the amino terminus of the *Paenibacillus* AOD, in which 25 amino acid residues have already been reported in Ref. 1, the amino acid sequence similarity was searched with a BLAST program. The $NH_2$-terminal amino acid sequence of this AOD was homologous to those of $Mn^{2+}$-SODs, and more than 60% of the amino acids were identical (Fig. 1). In addition, the highly conserved amino acid sequence (LP*X*LPY*XXXA*LEP and TM*X*IHH) found at $NH_2$-terminal region of many $Mn^{2+}$-SODs was also contained in that of the *Paenibacillus* AOD. Therefore, the relationship between AOD and SODs was then investigated.

**Assay of enzyme activity by color development method** AOD activity was measured by a color development method under standard assay conditions using 50 mM glycolaldehyde. The absorbance change per min at 555 nm linearly increased by increasing AOD amounts in the reaction mixture (Fig. 2A).

The SOD activity has so far been assayed by method 1 using xanthine oxidase and cytochrome *c*. However, the one drawback of this method was that SOD activity was measured by the reduction of absorbance. We therefore developed a new assay method for SOD activity as described in method 2, in which the rate of hydrogen peroxide formation was measured by the increased absorbance. The absorbance change per min at 555 nm linearly increased by increasing SOD amounts of the reaction mixture (Fig. 2B). These results indicate that SOD activity was also assayed by the increase of absorbance change at 555 nm, and that SOD activity obtained by this method can be compared to AOD activity by the same definition of the enzyme unit. We therefore used method 2 for the assay of SOD activity, while method 1 was used for the recognition of SOD activity in this report.

**SOD activity of *Paenibacillus* AOD** When a 0.65 m unit of *Paenibacillus* AOD was added to the reaction mixture following SOD activity measurement by method 1, the rate of absorbance increase at 550 nm was significantly slower than without AOD (Fig. 3A). Then, the same enzyme amount was added to the reaction mixture of method 2. An increase of absorbance was recognized at 555 nm (Fig. 3B). Thus, *Paenibacillus* AOD has SOD activity. In addition, this result indicated that 0.65 m unit of *Paenibacillus* AOD showed 1.02 m units of SOD activity. Thus, SOD activity of the *Paenibacillus* AOD was 1.6-fold higher than glycolaldehyde oxidase activity. The oxidase activities of this AOD on aldehyde alcohols such as aldotetrose and aldopentose were also assayed, since we have already reported that this AOD oxidized glycolaldehyde and glyceraldehyde (1). The aldotetrose such as D-threose, L-threose or D-erythrose were oxidized, but aldopentose were not (Table 1). Thus, the *Paenibacillus* AOD exhibited oxidase activity on glycolaldehyde,