Superoxide Dismutase in Ruminal Bacteria

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Of 13 species of anaerobic ruminal bacteria examined, 11 were found to contain measurable levels of superoxide dismutase activity. Four of five other strict anaerobic species studied for comparison were found to contain superoxide dismutase activity.

McCord et al. (10) proposed that the role of superoxide dismutase (SOD) in bacteria was to protect organisms from oxygen toxicity and that the lack of SOD was the enzymatic basis for anaerobiosis. They found that the strict anaerobic bacteria they studied had no SOD (10). We reported (R. S. Fulghum and J. M. Worthington, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, P158, p. 171) that certain strict anaerobic ruminal bacteria did show SOD activity and that exposure to oxygen increased SOD activity in some of these species but that SOD added to growth medium would not protect other strict anaerobes from media exposed to air. Since then, several laboratories have measured SOD levels in a number of species of strict anaerobic bacteria (3-7, 11-13). No published report has been made of SOD activity in anaerobic bacteria from the rumen of cattle. In this paper we report that SOD activity was found in 11 of 13 species of ruminal bacteria examined.

Ruminal bacteria and the other anaerobic bacteria were grown in artificial E medium, which contained the ingredients of the E medium of the Virginia Polytechnic Institute Anaerobe Laboratory (8) except that the fatty acid mix of Caldwell and Bryant (2), modified to reduce the acetic acid from 17 to 1 ml, was used in place of rumen fluid. The medium was prepared under prerreduced, anaerobically sterilized conditions (8) and inoculated and incubated under an oxygen-free mixture of 45% N2-45% CO2-10% H2. The medium used for Escherichia coli B was Trypticase soy broth (BBL Microbiology Systems) with glucose added to 1% and was inoculated and incubated in air with 5% CO2 or anaerobically under the oxygen-free gas mixture used for the strict anaerobic bacteria.

Ovine erythrocyte SOD was prepared by the method of McCord and Fridovich (9). The inhibition of the photochemical reduction of nitro blue tetrazolium (NBT) as described by Beauchamp and Fridovich (1) was used to assay for SOD activity in the cell washes and crude cell fractions. In this test, 50% inhibition is equivalent to 1 U of SOD activity (equivalent to 0.045 μg of the pure bovine erythrocyte SOD as described by Beauchamp and Fridovich (1)). Washes and fractions were prepared in a manner similar to the methods of Gregory et al. (6) except that EDTA was not used. The phosphate buffer cell wash, the cell extract (cytosol), and the cell debris fractions were individually analyzed for SOD activity. A color control reaction, containing all reagents but with water in place of the enzyme solution or sample, was also run. In each set of determinations, a quantity of ovine erythrocyte SOD sufficient to result in maximum inhibition in the NBT test was also run as a positive control. Samples (0.5 to 1.0 ml) of cell wash, cell extract (cytosol), and cell debris were assayed from each bacterial species for SOD activity. Determinations were made in duplicate in each set and from 2 to 10 sets of tests were performed with each species of bacterium. Data in Table 1 are averages of the different sets of tests and determinations within each test. Determinations were made with a Spectronic 20 colorimeter set at a wavelength of 560 nm. All inorganic reagents were Fisher analytical grade, and all organics were the highest grade offered by Sigma Chemical Co.

Percent inhibition was calculated as follows: percent inhibition = [(OD of color control — OD of sample)/(OD of color control)] × 100%, where OD is optical density.

The organisms assayed for SOD activity are listed in Table 1 along with the strain number and source of each. The data in this table show that all ruminal bacteria tested except for Bacteroides ruminicola subsp. ruminicola and Ruminococcus flavefaciens showed SOD activity in one or more fractions. Of the nonruminal bacteria tested for comparison, all except Clostridium novyi B showed SOD activity. Thus, our data show that 11 of 13 ruminal bacteria and 4 of 5 other bacteria growing under strict anaerobic conditions produce measurable levels of SOD activity. The data from the nonruminal strict anaerobic bacteria agree with previous reports in the literature (3-7, 11-13).

There are several possible advantages for ruminal bacteria to have SOD activity. These lie in the survival value of this activity during periods of oxygen exposure such as during transfer of the bacteria from the mother to the young animal initiating true ruminal activity and when the host animal drinks large quantities of water containing dissolved oxygen.

Beauchamp and Fridovich (1) have described the reactions of the assay using the photoreduction of NBT as follows. Illumination of solutions containing riboflavin, methionine, and NBT result in a linear accumulation of the blue formazan. In the absence of oxygen, SOD is without influence. However, under aerobic conditions, SOD inhibits the reduction of NBT. Thus, photoreduced riboflavin can reduce NBT by a pathway that utilizes superoxide radical (O2·−) as an electron-carrying intermediate, and in the presence of oxygen, SOD inhibits this reaction. The aerobic pathway is the preferred reaction. Anaerobic photoreduction of NBT is unaffected by SOD, whereas the aerobic photoreduction rate is inhibited by 95% with 1.3 μg of SOD per ml. The maximum inhibition achieved by the addition of crude cell extracts is less than that of pure SOD, about 70% as compared with 95%. This is due to elements of electron transport which provide an oxygen-independent route of electron transport from photoreduced riboflavin to NBT.

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This does not bar the use of the photoreduction of NBT inhibition assay to crude extracts, since a unit of SOD activity could be defined as the amount needed to cause 50% inhibition (1). Thus, although 50% inhibition of NBT reduction is equivalent to one unit of activity, the relationship is not linear out to 100%. In our study, the maximum average inhibition found in crude extracts was 72% (the range with this organism was 68 to 76%), whereas our semipurified ovine SOD inhibited the reaction by 93% (range, 89 to 98%). Therefore, one problem with the NBT photoreduction inhibition test is that it cannot measure SOD activity quantitatively and there can be no correlation of the enzyme activity to the amount of protein in the crude extracts used. Nevertheless, the NBT photoreduction inhibition assay can be used for screening for SOD activity in crude extracts.

There are two other points regarding the NBT photoreduction assay that need to be considered. Any light-absorbing materials, e.g., hemoglobin, could also inhibit the photoreduction of NBT by absorbing light needed to reduce the riboflavin. However, if no great amount of light-absorbing substances are present, the assay is practical within the limits mentioned above. The other point is that the reactions that dismutate $O_2^-$ are enzymatic reactions that are reversible. Since molecular oxygen is an end product of the dismutation of $O_2^-$, high concentrations of oxygen may reverse the reactions. In the aqueous environment of the NBT photoreduction assay, the oxygen concentration is sufficient for the production of $O_2^-$ but not sufficient for the reverse reaction. Reversal of the reaction could be a problem in electrophoretic assays with thin gels (I. Fridovich, personal communication).

The tendency of the blue formazan to form a fine precipitate rather than a uniform blue solution makes precise colorimetric readings difficult. Fridovich (personal communication) now suggests the addition of a small amount of detergent to minimize the precipitation of the blue formazan. Washing the cells before analysis probably disrupts a proportion of the cells of most of the species in this study. We did not routinely study all preparations; however, spot checks of materials in pellets centrifuged from the cell wash showed many intact cells when viewed by phase-contrast microscopy. Some of our data would support the argument that disruption of at least the outer membranes in Bacteroides ruminicola subsp. brevis occurred, since the bulk of the SOD activity is found in the wash. Disruption probably occurred to a lesser extent in the other species, in which activity was distributed among all fractions; however, in Eubacterium ruminantium most of the activity was found in the cytosol.

In conclusion, we have presented data that show that 11 of 13 species of ruminal bacteria as well as 4 of 5 other species contain measurable levels of SOD activity.

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**LITERATURE CITED**