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Some Properties of Superoxide Dismutase from *Dirofilaria immitis*

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Active oxygen scavenging enzymes, such as catalase and glutathione peroxidase, are very important in parasites, as they protect against oxygen-mediated damage caused by normal metabolic and other processes. Superoxide dismutase (SOD), which catalyzes the disproportion of the superoxide anion, is an active oxygen scavenging enzyme which is found in many protozoan and helminthic parasites (Docampo and Moreno, 1984). *Dirofilaria immitis* in the dog heart would also be exposed to an oxygen-mediated damage. The presence of SOD in *D. immitis* adult worms and microfilaria has already been reported by Callahan *et al.* (1988). However, detailed enzyme property has not yet been described. The present work was undertaken to determine the existence of SOD in adult *D. immitis* by highly sensitive methods using nitroblue tetrazolium (NBT) and *cypridina* luciferin analog (MCLA) as a reactant with O_2^-. Furthermore, some of the properties of the SOD are discussed.

Dog erythrocyte SOD was prepared from dog blood cells according to the method described by McCord and Fridovich (1969). Bovine erythrocyte SOD and xanthine oxidase were purchased from Sigma Chemical Co. *D. immitis* adult worms were collected from dog hearts, lyophilized after repeated washings with saline and then stored at −30°C until use. To study intracellular localization of SOD, live worms were used immediately after washing with saline. SOD was mainly assayed by NBT method described by Sun *et al.* (1988), or by MCLA method described by Kimura and Nakano (1988). SOD activity was expressed as the amount of bovine erythrocyte SOD required for inhibiting blue formazan color development or chemiluminescence. Protein concentration was measured by the Lowry method (Lowry *et al.*, 1951). SOD and other proteins were separated by polyacrylamide gel electrophoresis using a non SDS gradient gel (8–16%) purchased from TEF Co. Japan.

Lyophilized adult worms were cut into small pieces with scissors, suspended in 0.3 M potassium phosphate buffer, pH 7.8 and stored in a refrigerator overnight. The suspension was homogenized for 15 min with a hiscotrone (Nihon Seimitsu Kogyo KK, Japan) in ice water. After sonication (Nippon Seiki Co., Japan) at 150 mA for 15 min (5 min × 3 times), the homogenate was centrifuged at 13,000 g for 30 min. The supernatant was concentrated by ultrafiltration using Visking cellulose tubing 8/32. The precipitate, which appeared during concentration, were removed by centrifugation. SOD and protein in the supernatant (crude extract) were measured. As shown in Table 1, the SOD concentration corresponded to 0.1% of the total protein concentration in the crude extract. When 5 mM potassium cyanide was added to the crude extract and incubated for 30 min at 37°C, SOD activity was completely abolished, indicating that

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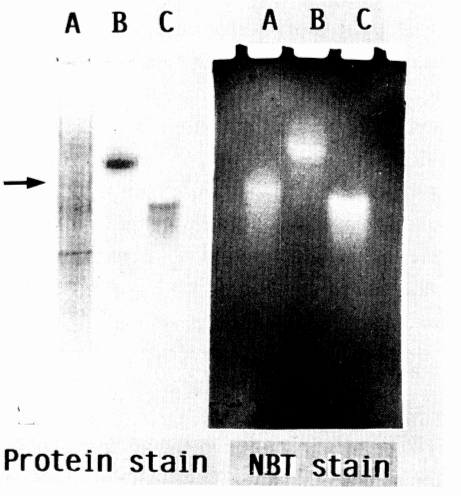
Table 1 The superoxide dismutase content in the crude extract from 1 g of *D. immitis* lyophilized adult worms determined by NBT and MCLA methods

SOD measurement Method	Total Protein (mg)	Total SOD (ng)	SOD/Protein (%)	KCN subtraction (%)
NBT	100.9	12.8	0.117	100
MCLA	109.4	10.6	0.097	86

SOD in *D. immitis* adult worms is cyanide-sensitive in keeping with common copper, zinc-SOD.

To compare with electrophoretic properties of three samples, SOD in the crude extract, bovine and dog erythrocyte SOD, each other, these samples were run on polyacrylamide gel plate and stained with Coomassie brilliant blue for protein and with NBT for SOD (Beauchamp and Fridovich, 1971). As shown in Fig. 1, several protein bands were stained and a single SOD band which migrated near bovine erythrocyte SOD, but faster than dog erythrocyte SOD was revealed by NBT staining.

To study intracellular localization of SOD in *D. immitis,* 3 g of live worms, suspended in 0.01 M Tris-HCl buffer containing 0.25 M sucrose (Tris-sucrose buffer) at pH 7.2, were cut into small pieces and homogenized with a hiscotrone. The homogenate was sequentially centrifuged as follows; 600 g for 10 min, 5,500 g for 20 min, 11,000 g for 30 min and 100,000 g for 90 min. Each precipitate was resuspended in Tris-sucrose



A: *D. immitis* crude extract
B: Dog erythrocyte SOD
C: Bovine erythrocyte SOD

Fig. 1. Protein stain and NBT stain for superoxide dismutase (SOD) of the gel after polyacrylamide gel electrophoresis of *D. immitis* crude extract. The arrow in the protein stained gel indicates the SOD band of *D. immitis* crude extract.

Table 2 Intracellular localization of the superoxide dismutase of 2.5 g in *D. immitis* live adult worms

Fractions tested	Total vol (ml)	Total protein (mg)	Total SOD content (μg)	(%)
Crude extract	39.1	113.4	97.8	100
600g ppt	10.0	60.0	10.0	9.3
5,500g ppt	4.0	3.2	0.3	0.3
10,000g ppt	1.3	2.9	ND	ND
100,000g ppt	1.3	2.7	ND	ND
100,000g sup	22.5	45.0	96.8	90.4

ND: not detected, ppt: precipitate, sup: supernatant

buffer and sonicated at 150 mA for 3 min. The SOD activities in the sonicated samples and the clear supernatant from the 100,000 g-centrifugation were assayed by NBT method. As shown in Table 2, more than 90% of the total SOD was found in the 100,000 g-supernatant, while only 9.3% was detected in 600 g-precipitate. Little or no SOD activity was detected in the other fractions.

Three types, copper- zinc-, manganese- and iron-SOD, have been identified in various organisms (Docampo and Moreno, 1984), Cu, Zn-SOD is the most common one and is located predominantly in the cytosol and in the mitochondrial intermembrane spaces of eukaryotic cells. This enzyme is very sensitive to cyanide. Mn-SOD found in prokaryotic and eukaryotic cells is abundantly rich in the matrix space of mitochondria and is cyanide-insensitive enzyme. Fe-SOD identified in bacteria, protozoa and some higher plants is also insensitive to cyanide. In parasites, the presence of SOD was first demonstrated in protozoa, *Trichomonas foetus* (Lindmark and Mullar, 1974), then in *Trypanosoma cruzi* and *T. brucei* (Opperdoes *et al.*, 1977), *Leishmania tropica, L. donovani* (Meshnick *et al.*, 1983), *Toxoplasma gondii* and *Entamoeba histolytica* (Murray *et al.*, 1981). Furthermore, it has also been found in the helminths, *Trichinella spiralis* (Rhoads, 1983), *Fasciola hepatica* (Barrett, 1980), and *Hymenolepis diminuta* (Paul and Barrett, 1980) etc. Although detailed properties of these SOD have not been studied yet except for those of *T. spiralis,* all are classified as Cu, Zn-SOD because of their cyanide-sensitivity. As shown in this report, *D. immitis* SOD was highly sensitive to cyanide and about 90% of the total SOD activity was found in the 100,000 g-supernatant. These results suggest that *D. immitis* SOD is located in the cytosol and that it belongs to the Cu, Zn-type. Callahan et al. (1988) have also proposed that the SODs of *Onchocerca cervicalis* and *D. immitis* are of the Cu, Zn-type. On the other hand, Suthipark et al. (1982) have reported that the SOD isolated from *Plasmodium berghei* is electrophoretically indistinguishable from the host-SOD, and then malarial SOD is entirely of host origin. The electrophoretic mobility of the SOD of *D. immitis* adult worms was completely different from that of the dog erythrocyte SOD. Accordingly, we consider that the *D. immitis* SOD is intrinsic in these worms.

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