

# Superoxide Dismutases

## I. OCCURRENCE IN HIGHER PLANTS<sup>1, 2</sup>

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### ABSTRACT

Shoots, roots, and seeds of corn (*Zea mays* L., cv. Michigan 500), oats (*Avena sativa* L., cv. Au Sable), and peas (*Pisum sativum* L., cv. Wando) were analyzed for their superoxide dismutase content using a photochemical assay system consisting of methionine, riboflavin, and *p*-nitro blue tetrazolium. The enzyme is present in the shoots, roots, and seeds of the three species. On a dry weight basis, shoots contain more enzyme than roots. In seeds, the enzyme is present in both the embryo and the storage tissue. Electrophoresis indicated a total of 10 distinct forms of the enzyme. Corn contained seven of these forms and oats three. Peas contained one of the corn and two of the oat enzymes. Nine of the enzyme activities were eliminated with cyanide treatment suggesting that they may be cupro-zinc enzymes, whereas one was cyanide-resistant and may be a manganese enzyme. Some of the leaf superoxide dismutases were found primarily in mitochondria or chloroplasts. Peroxidases at high concentrations interfere with the assay. In test tube assays of crude extracts from seedlings, the interference was negligible. On gels, however, peroxidases may account for two of the 10 superoxide dismutase forms.

### MATERIALS AND METHODS

**Plant Material.** Seeds and seedlings of corn (*Zea mays* L., cv. Michigan 500), oats (*Avena sativa* L., cv. Au Sable), and peas (*Pisum sativum* L., cv. Wando) were utilized.

Seeds were treated with 0.3% (w/v) captan 80W for 5 min and germinated in plastic flats containing 3 cm Turface (Turf Supplies Co., Taylor, Mich.) at the bottom and 3 cm vermiculite at the top. The seedlings were grown for 10 days in a growth chamber under 26 C, 25 kilolux of light, and a 12-hr photoperiod. Half-strength Hoagland solution was provided once after 6 days and distilled H<sub>2</sub>O throughout growth as needed.

Embryos and scutella were excised from seeds which had been allowed to imbibe at 25 C for 10 hr and were rinsed three times with distilled H<sub>2</sub>O. Endosperm was obtained from dry corn and oat seeds from which the embryo-bearing end had been removed. Endosperm was drilled from the cut surface by using a size 60 wire bit fitted on a battery operated drill. The hulls of oat seeds were removed, unless the seeds were to be germinated.

**Preparation of Extracts.** The tissues were thoroughly ground with a cold mortar and pestle in an ice bath, until no fibrous residue could be seen. The grinding medium (4-6 ml/g fresh weight) consisted of 0.1 M K-phosphate and 0.1 mM EDTA (pH 7.8), plus homogenizing glass beads. The homogenate was centrifuged twice at 13000g for 10 min in a Sorvall RC2-B refrigerated centrifuge at 0 to 5 C. The supernatant, hereafter referred to as crude SOD extract, was used for electrophoresis and for determination of the SOD content in the tissue.

The water-soluble protein content of all crude SOD extracts was determined by the method of Lowry *et al.* (14) after precipitation with 10% trichloroacetic acid. Bovine serum albumin was used as a standard. Extracts were diluted 10 times for SOD assays. To the extracts for electrophoresis 1 M sucrose was added to increase density and avoid mixing in the reservoir.

**Isolation of Organelles.** Mitochondria were isolated by the method of Bonner (5) and chloroplasts by the method of Smillie (21) from shoots of 7-day-old seedlings grown in the dark and light, respectively. The organelle pellets were washed twice by resuspending in wash media and recentrifuging. The washed pellets were finally resuspended in small volumes of 0.05 M K-phosphate (pH 7.8), and sonicated for 3 min. After dialysis against changes of the same buffer and centrifugation, the supernatants were used for electrophoresis.

**Electrophoresis.** Polyacrylamide gel electrophoresis of the crude SOD extracts was performed according to Davis (6). The migration was from the cathode to the anode. Each extract was applied to the gels at various concentrations ranging from 50 to 300  $\mu$ g of protein, or 5 to 20 units of enzyme. A current of 1 mamp/gel was applied during migration of the bromphenol blue marker on the spacer gel. Electrophoresis was continued under 2 mamp/gel until the marker had migrated approximately 10 cm on the resolving gel.

The superoxide dismutases were localized by the photochemi-

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Superoxide dismutases are metalloproteins catalyzing the dismutation of the superoxide free radical (O<sub>2</sub><sup>-</sup>) to molecular oxygen and H<sub>2</sub>O<sub>2</sub>. This enzymic activity was first described by McCord and Fridovich (17) with a cupro-zinc protein (erythrocyuprein) from bovine erythrocytes. Similar cupro-zinc proteins with SOD<sup>3</sup> activity were subsequently isolated from various eukaryotic sources (8). Manganese-containing proteins with SOD activity were later found in prokaryotes and in mitochondria of eukaryotes (8). Iron proteins from *Escherichia coli* (10) and algae (15) were recently shown to possess SOD activity.

Cupro-zinc SOD has already been isolated from tissues of higher plants: pea seeds (19), spinach leaves (2, 15), and wheat germ (4). Isozymes have also been reported for wheat and spinach cupro-zinc enzyme (4, 15). A cyanide-resistant enzyme from wheat germ has been described as manganese-SOD (4). Occurrence of manganese enzyme in spinach chloroplasts has been reported (15), but is not certain (7). There is no report of iron enzyme in higher plants.

A comparative study of the SOD distribution in plants has not been conducted thus far. Distribution of the enzyme within the plant and intraspecific differences may be of interest and were the objective of the present study.

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<sup>2</sup> This paper represents part of the Ph.D. dissertation of C. N. G.

<sup>3</sup> Abbreviations: SOD: superoxide dismutase (EC 1.15.1.1); NBT: *p*-nitro blue tetrazolium chloride; HRP: horseradish peroxidase.

cal procedure of Beauchamp and Fridovich (3) as modified by Weisiger and Fridovich (22). The gels were first soaked in a solution of NBT, then immersed in a solution of riboflavin and tetramethylethylenediamine, and illuminated in tubes containing K-phosphate and EDTA at pH 7.8. The stained gels were photographed on Kodak Panatomic X 120 film using a 25 A red filter, or scanned with a Gilford model 220 gel densitometer.

**Enzyme Assay.** All extracts were assayed for SOD activity photochemically, using the assay system consisting of methionine, riboflavin, and NBT (3). The photochemical procedure was chosen as being independent of other enzymes and proteins and, therefore, more reliable in the case of crude extracts than enzymic assay systems (17).

The original assay described by Beauchamp and Fridovich (3) was modified. The reaction mixture was composed of 1.3  $\mu\text{M}$  riboflavin, 13 mM methionine, 63  $\mu\text{M}$  NBT, 0.05 M sodium carbonate (pH 10.2), and the appropriate volume of extract. Distilled  $\text{H}_2\text{O}$  was added to bring to the final volume of 3 ml. The mixtures were illuminated in glass test tubes selected for uniform thickness and color. Identical solutions that were not illuminated served as blanks.

The apparatus devised for exposing the tubes to light was composed of a rotating test tube holder (Rayonet MGR-100, Southern New England UV Co.) immersed in water in a cylindrical glass container thermostated at 25 C by a Forma 2095 refrigerated and heated water circulator. A circular fluorescent lamp (Sylvania, FC 12 T 10-CW-RS) was attached on the outside wall of the water bath and the entire assembly was fitted in a box lined with aluminum foil. The reaction was initiated and terminated by turning the light on and off. There was no detectable amount of the reaction occurring under room light during preparation of the solutions and spectrophotometric measurements.

The initial rate of the reaction was determined as increase of absorbance at 560 nm. Under the described conditions, the increase of absorbance in absence of SOD was 0.100 absorbance unit/5 min and was linear up to 15 min. In the presence of SOD, the reaction was inhibited and the amount of inhibition was used to quantitate the enzyme. Each extract was assayed twice and the results varied less than  $\pm 0.005$  absorbance unit/5 min.

Dialysis and gel filtration (Sephadex G-50) of crude SOD extracts indicated no significant interference of small molecules with the assay.

**Interference Experiments.** Crude extracts used in these experiments were prepared from shoots and roots of 10-day-old seedlings as previously described. Horseradish peroxidase (A grade) was purchased from Calbiochem. Peroxidase activity was determined with the guaiacol test (20). Gels were stained for peroxidase localization according to Hart *et al.* (12). Crude extracts were heated in portions of 1 ml in Corex 17-ml centrifuge tubes kept in a boiling water bath (95–97 C) for 0.5 to 20 min. Precipitated protein was removed by centrifugation.

## RESULTS AND DISCUSSION

**Enzyme Quantitation.** Beauchamp and Fridovich (3) defined 1 unit of SOD as the amount that inhibits the NBT photoreduction by 50% and quantitated the enzyme on basis of the per cent inhibition it caused. Per cent inhibition and SOD concentration were not linear, however. Asada *et al.* (1), using crystalline spinach SOD and the xanthine/xanthine oxidase assay system (17), established a linear relationship between SOD concentration and the  $V/\nu$  ratio fitting the equation:

$$V/\nu = 1 + K'[\text{SOD}] \quad (1)$$

where  $V$  and  $\nu$  represent the rate of the assay reaction in absence and in presence of SOD, respectively. This linear relationship was also observed in the present study with crude extracts and the photochemical assay system (Fig. 1).

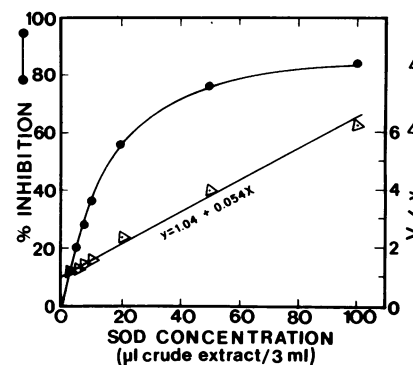


Fig. 1. Inhibition of the NBT photoreduction versus concentration of crude SOD from corn seeds. Crude extract was prepared from 10 corn seeds homogenized with 15 ml of 0.1 M K-phosphate (pH 7.8). Per cent inhibition is not linear with SOD concentration. Linearity ( $r = 0.995$ ) is obtained by plotting  $V/\nu$  ratio against SOD concentration,  $V$  and  $\nu$  representing the rate of the reaction in absence and presence of enzyme, respectively. Linearity between  $[\text{SOD}]$  and  $V/\nu$  was also confirmed with crude extracts from seeds, shoots, and roots of both peas and oats. The intercept of all lines was  $1.00 \pm 0.03$ , and the slope  $0.049 \pm 0.005$ . Linearity was maintained up to 70% inhibition.

If the SOD unit is redefined, the equation of Asada *et al.* (1) allows convenient and accurate determination of SOD activity. Equation 1 can be rewritten as:

$$K'[\text{SOD}] = (V/\nu) - 1 \quad (2)$$

At 50% inhibition of the assay reaction, the product  $K'[\text{SOD}]$  equals unity. If the SOD unit is redefined as the amount of enzyme for which  $K'[\text{SOD}]$  equals unity, then SOD activity can be determined directly from the  $V/\nu$  ratio according to the equation:

$$\text{SOD units/ml} = [(V/\nu) - 1] (\text{dilution factor}) \quad (3)$$

One unit thus determined is equivalent to 3.03 units as described by McCord and Fridovich (17).

The constant  $K'$ , which corresponds to the slope of the line in Figure 1, is a function of the NBT concentration and the affinities of SOD and NBT for  $\text{O}_2 \cdot^-$  (1). Under the assay conditions used in this study, the  $K'$  value determined by using crude extracts was 0.049 (expressed in  $\mu\text{l}^{-1}/3 \text{ ml}^{-1}$ ). The  $K'$  value determined with pure SOD from bovine erythrocytes (Sigma) was 0.020. This value multiplied by a factor of approximately 2, since SOD from plant sources has twice the specific activity as SOD from mammalian sources (19), becomes similar to the value of  $K'$  obtained from crude extracts. By using pure bovine SOD and assuming that the mol wt of plant SOD is 30,000 (19), it was further determined that 1 unit of SOD activity in the crude extracts was equivalent to an enzyme concentration of 3.66  $\eta\text{M}$ .

**Superoxide Dismutase Content of Plant Organs and Tissues.** Both enzyme assays and electrophoresis established that extractable SOD was present in seeds and various seed parts, roots, leaves, and shoots of the three species.

In seeds, SOD was present in both the embryo and the storage tissue (Table I). In this study, the units of enzyme/seed were determined photochemically from extracts of whole seeds, embryos, and storage tissue. It was also established that during imbibition of seeds, from 0 to 25 hr, the total activity of enzyme/seed as well as the activity/embryo and storage tissue were not altered. A fixed amount of SOD is always present in the embryo and the storage tissue, and no *de novo* synthesis or activation needs to occur upon imbibition (Table I).

Most of the enzyme in oat and pea seeds was found in the storage tissue; the oat endosperm contained 62% and the pea cotyledons 82% of the total activity (Table I). By contrast, in the corn seed, more enzyme was found in the embryo than in the storage tissue. This may be attributed to the fact that the corn

Table I. Superoxide Dismutase Content of Seeds and Seed Parts.

Each value is the average of 8 non-significantly different values obtained from seeds soaked in water for 0, 5, 10, and 25 hr (2 samples each).

Species	Whole Seed <sup>1</sup>	Embryo <sup>2</sup>	Storage Tissue <sup>3</sup>
<u>SOD units/seed</u>			
Corn	40.7	26.7	17.7
Oats	20.5	8.0	12.7
Peas	792.2	142.0	652.0
<u>SOD units/g dry wt</u>			
Corn	194	1057	96
Oats	884	4819	565
Peas	4681	39776	3739

<sup>1</sup>Purification of the enzyme from pea seeds did not indicate any interference of impurities with the crude extract assays.

<sup>2</sup>The scutellum of corn and oats is included in the embryo.

<sup>3</sup>Storage tissue: the remaining part of the seed after removal of the embryo.

Table II. Superoxide Dismutase Content of 10-day-old Seedlings.

Each value is the average of two samples extracted and assayed independently. The enzyme has been purified from pea seeds and corn seedlings and interferences with the assay are negligible for crude extracts from seedlings of the three species.

Species	Organ	Dry wt (mg/plant)	Protein (mg/g dry wt)	Units/Plant	Units/mg dry wt	Units/mg protein
Corn	Shoot	143	58	579	4.0	69
Corn	Root	146	21	124	0.9	41
Oats	Shoot	13	133	90	6.9	52
Oats	Root	13	32	32	2.5	76
Peas	Shoot	67	208	305	4.6	22
Peas	Root	62	51	127	2.0	20
L.S.D. at 0.05	...		19	206	1.7	20

embryo (including the scutellum) constitutes a considerably larger portion of the whole seed than the oat or pea embryo. On a dry weight basis, the embryo of all three species contains approximately 10 times more enzyme than the storage tissue. Therefore, the embryo is richer in SOD regardless of containing more or less enzyme than the storage tissue on a per seed basis. The occurrence of the enzyme in all seed parts, and more importantly in the endosperm, is of particular interest. If the proposed biological role for this enzyme (9, 11) is accepted, then its distribution may demonstrate that  $O_2 \cdot^-$  can be formed in seeds and may be involved in aging and reduced viability of seeds, as suggested by Pammenter *et al.* (18).

The enzyme was found in both shoots and roots of the seedlings (Table II). In this study, the seedlings were grown up to the stage that the shoots and roots of each species accumulated the same amount of dry matter at harvest. On a dry weight basis, the shoots of all three species contain more protein and SOD than the roots. On a protein basis, the shoots and roots of the two grasses contain two to three times more enzyme than the shoots and roots of peas. On a dry weight basis, seeds and shoots of peas contain the same amount of enzyme, whereas oat and corn seeds contain considerably less enzyme than the corresponding shoots.

**Multiple Forms of SOD.** Electrophoresis indicated that the SOD activity of the crude extracts is composed of a number of distinct bands. Ten SOD active bands were obtained from a mixture of the extracts of the three plant species in a single electrophoretic run. These bands are numbered in order of increasing relative mobility in Figures 2 and 3.

Within a species, the same active bands with apparent quantitative differences were obtained from seeds, roots, and leaves (Figs. 2 and 3). From the different species, a different number of bands were obtained. In corn, seven bands were observed, in contrast to only three in oats and peas. Only bands of high relative mobility were obtained from oats and peas, whereas corn also contained bands of intermediate and low relative mobilities. High concentrations of oat and pea extracts did not produce additional bands, even though the observed bands overlapped. The corn bands of intermediate and low relative mobility were also observed at considerably lower concentrations of extract. The same banding patterns were obtained under various extraction conditions, such as pH 6 to 8, K-phosphate concentrations 0.005 to 0.4 M, and presence or absence of 0.1 mM EDTA. Dialysis and ammonium sulfate or acetone fractionation of the crude extracts did not alter the banding patterns. Freeze-thawing and aging in the cold resulted in additional minor bands.

The above 10 bands may correspond to different proteins with SOD activity, or different isozymes of a single protein, or a combination of both. Proteins containing cupro-zinc, manganese, or iron have already been described to function as superoxide dismutases (8). Isozymes of cupro-zinc SOD have also been reported (4, 15).

Cupro-zinc enzymes are sensitive to cyanide, whereas manganese enzymes are resistant to cyanide and sensitive to treatment with a chloroform-ethanol mixture, thus allowing their discrimination on gels (4, 22). The only enzyme that was found resistant to cyanide was SOD 5 of corn (Fig. 4). It seems likely that SOD 5 is a manganese enzyme, all the remaining being isozymes of cupro-zinc SOD.

Quantitative differences of the SOD bands among organs or tissues are evident from the "band area." Preliminary experiments established that band area is proportional to the enzyme concentration at the amount of extracts used in these studies. The following differences between seed parts can be seen in Figure 2. Corn endosperm does not contain any detectable amount of SOD 1, and compared to the other seed parts, is lower in SOD 3 and 4, but higher in SOD 2. A similar situation occurs in oat seeds; the endosperm is low in SOD 6 and high in SOD 8, exactly the opposite of the embryo. The remaining part of the oat seed after the removal of the embryo, which includes aleurone and endosperm, is high in both SOD 6 and 8, suggesting that the aleurone must be similar to the embryo. There is no significant difference between pea embryo axes and cotyledons.

The localization of some of the SOD enzymes in organelles was explored as a possible explanation of the above differences. Mitochondria were isolated from etiolated seedlings and the extracts from them were subjected to electrophoresis at various concentrations (Fig. 4). The SOD enzymes in which endosperm is rich (SOD 2 and 8) were not detected in mitochondrial extracts. The SOD enzymes in which embryo, aleurone, or cotyledons are rich (SOD 1, 3, 4, 5, and 6) were major bands in mitochondria. A possible explanation for the above differences may be the fact that SOD 1, 3, 4, 5, and 6 are primarily but not exclusively localized in mitochondria. Embryo, scutellum, aleurone, and cotyledon tissue contain a large number of mitochondria, in contrast to the endosperm.

Major similarities between the SOD pattern of the roots (Fig. 3) and that of the embryos (Fig. 2) are in agreement with the localization of certain SOD forms in mitochondria. The leaves, compared to both seeds and roots, contain SOD 9 or 10 as the major bands. Chloroplasts isolated from leaves of young seedlings contained more of these two enzymes than mitochondria (Fig. 4). This difference in SOD pattern between mitochondria and chloroplasts may be the reason for the difference in SOD pattern between leaves and roots.

**Interferences with Enzyme Activity in Crude Extract Assays.** Experiments were conducted to study interference of impurities

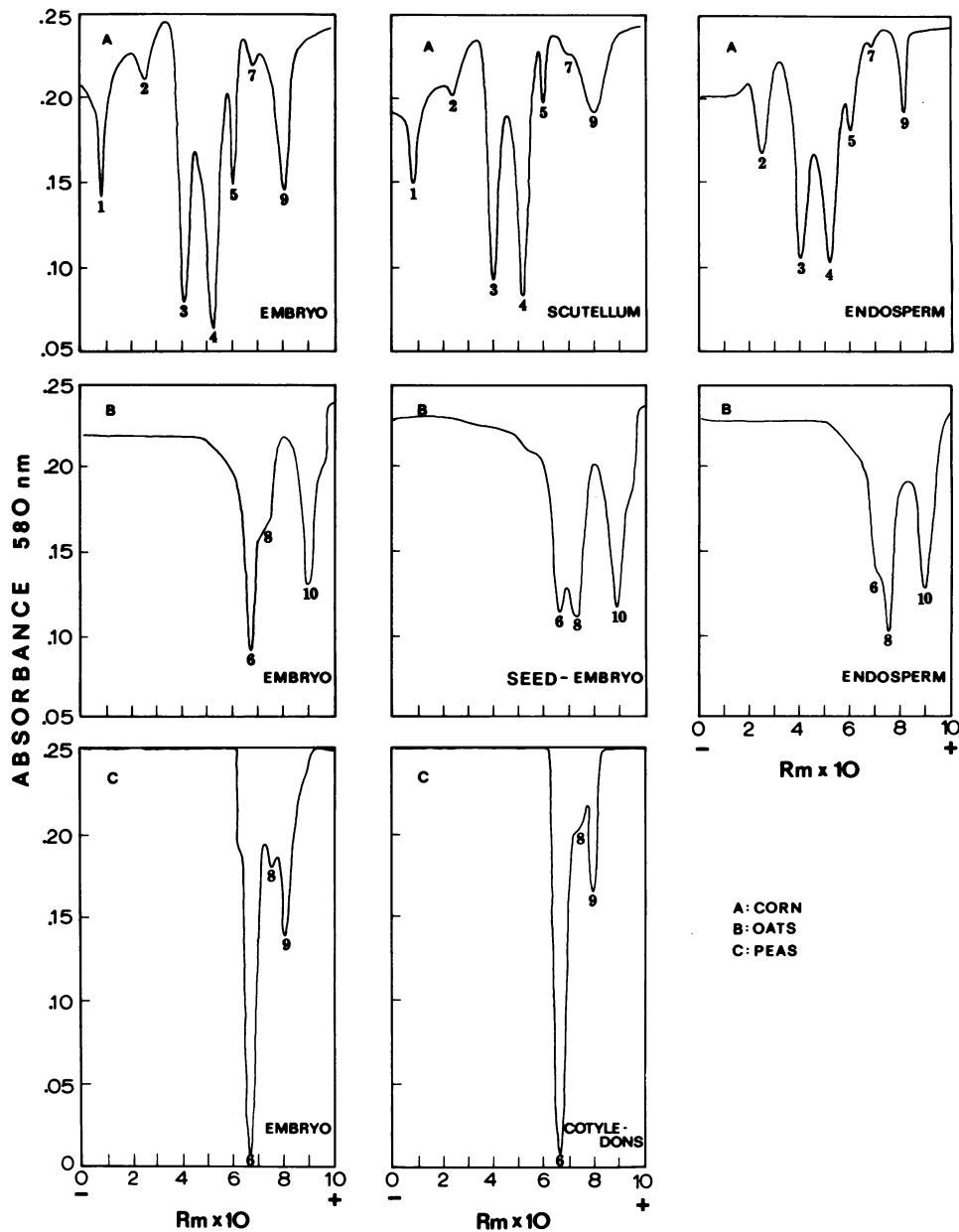


FIG. 2. Densitometer tracings of gels showing the seed superoxide dismutases (anodic forms only). Extracts from the indicated seed parts (6 ml/g) were used at 10  $\mu$ l/gel, except for the extracts from oat and corn endosperm, which were used at 50 and 100  $\mu$ l, respectively. The enzymes, which appeared as achromatic bands on blue-stained gels, are shown here as negative peaks, and are numbered in order of increasing relative mobility. The relative mobilities of the bands, obtained from extracts of the three species applied on gels singly and in combination, suggested that SOD 6 and 8 are common to peas and oats, while SOD 9 is common to peas and corn. Rm: relative mobility (migration distance of band/migration distance of marker).

in the crude extracts with the enzyme activity. Thus, a basis could be provided for assessing the reliability of the data presented in the previous sections.

Interference by impurities which scavenge  $O_2^{\cdot -}$  can be estimated from the value of the constant  $K$  determined as described under "Enzyme Quantitation." Such impurities should lead to a  $K'$  value higher than expected. Under the assay conditions used in this study, interferences were insignificant. This is further supported with results presented here.

Complete loss of the activity in crude extracts was achieved when 3 mM KCN was included in the assay mixture (Table III). Chloroform-ethanol treatment of the extracts caused an approximately 5% loss of activity only in the case of corn. Cupro-zinc SOD is inactivated by cyanide and manganese SOD by the

chloroform-ethanol treatment (22). Even though complete loss of activity was obtained with potassium cyanide, other enzymes may interfere with the assay since cyanide is not a specific inhibitor of SOD. The results from this experiment indicate that there is no interference by nonenzymic impurities. Dialysis of the crude extracts has indicated no interference by small mol wt impurities.

Peroxidases might interfere with the photochemical assay. Horseradish peroxidase retarded the accumulation of blue formazan, thus exhibiting an SOD-like activity (Fig. 5). This activity was equivalent to 40.6 SOD units/mg of HRP electrophoresis of HRP and peroxidase activities yielded three weak SOD bands which were of identical relative mobility with the three most intense peroxidase bands.

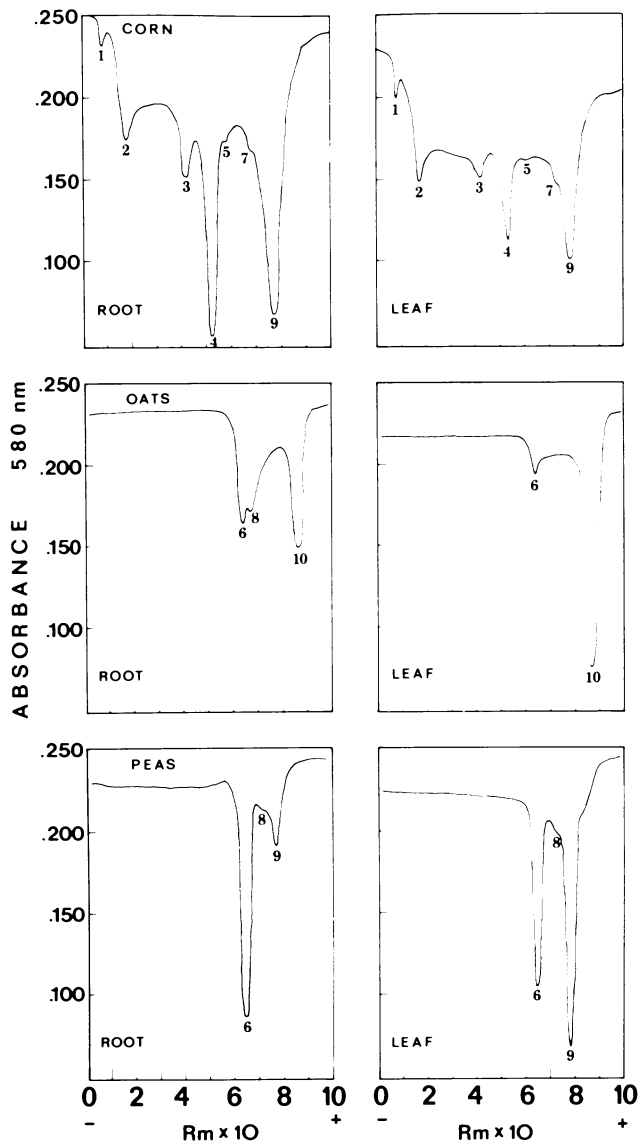


FIG. 3. Densitometer tracings of gels showing the seedling superoxide dismutases (anodic forms only). Extracts from roots and leaves were used at 50  $\mu\text{g}$  of protein/gel, except for the extract from corn leaves, which was used at 100  $\mu\text{g}$  of protein. The enzymes, which appeared as achromatic bands on blue-stained gels, are shown here as negative peaks, and are numbered in order of increasing relative mobility. Rm: relative mobility (see Fig. 2).

The SOD-like activity of HRP, therefore, is due to the peroxidase and not to contamination with SOD.

Peroxidase is not likely to inhibit the photoreduction of NBT, but is likely to catalyze the oxidation of the blue formazan formed. This was supported by the observation that blue formazan was bleached upon incubation with HRP and  $\text{H}_2\text{O}_2$  in the light or dark. Hydrogen peroxide, which is required for the action of the peroxidase, is produced in the SOD assay system. In absence of SOD,  $\text{H}_2\text{O}_2$  is produced at low concentrations from the spontaneous dismutation of  $\text{O}_2 \cdot^-$  (13). Higher concentrations of  $\text{H}_2\text{O}_2$  are produced in the presence of SOD from both spontaneous and enzymic dismutation of  $\text{O}_2 \cdot^-$  (17). When purified pea SOD and HRP were both included in the photochemical assay, the SOD-like activity of the peroxidase was doubled (Fig. 5). Increasing the SOD concentration did not further increase the activity of the peroxidase. This suggests that low  $\text{H}_2\text{O}_2$

concentration was limiting the action of peroxidase in absence but not in the presence of SOD.

Attempts were made to estimate the extent to which peroxidase in the crude extracts could interfere with the SOD assays. Peroxidase and SOD activities were both determined in crude extracts of corn, oats, and peas. Peroxidase activity as determined with the guaiacol test (20) varied from 263 to 9022 units/ml. As estimated from Figure 5, peroxidase at these concentrations would account for less than 2.5 SOD units/ml, which represents 3 to 12% of the total SOD units photochemically determined in oat and pea crude extracts, respectively. It was further observed that peroxidase could be easily inactivated by heating the crude extracts in a boiling water bath; SOD was relatively heat-stable. The peroxidase of all crude extracts was completely inactivated after heating for 1.5 min. This was not

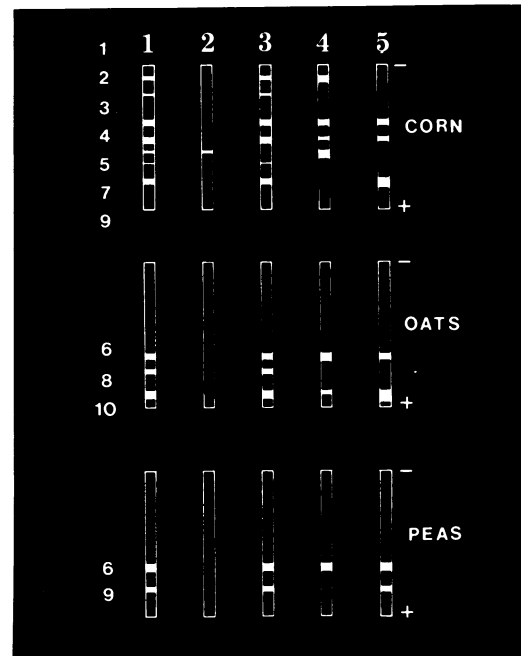


FIG. 4. Sensitivity to cyanide and chloroform-ethanol treatments, and organelle specificity of the superoxide dismutases. Treatments (numbers at the top) are as follows: 1: leaf extract; 2: leaf extract and cyanide treatment; 3: leaf extract and chloroform-ethanol treatment; 4: mitochondrial extract from etiolated seedlings; 5: chloroplast extract. Potassium cyanide at 1 mM was included in the staining solutions for treatment 2. For treatment 3, the extracts were mixed with chloroform (0.15 volume) and ethanol (0.25 volume), and centrifuged before applied on the gels. The enzyme bands are numbered (on the left) as in Figures 2 and 3.

Table III. Effect of Potassium Cyanide and Chloroform-Ethanol Treatments on SOD Activity of Crude Extracts.

Extract	Treatment	SOD Activity	
		Units/ml	%
Corn	None	50.0	...
	Chloroform-ethanol	42.3	3.4
	KCN 1 mM	10.0	80.0
	KCN 3 mM	0.0	100.0
Oats	None	62.3	...
	Chloroform-ethanol	62.1	0.0
	KCN 3 mM	0.0	100.0
Peas	None	31.5	...
	Chloroform-ethanol	31.5	0.0
	KCN 3 mM	0.0	100.0

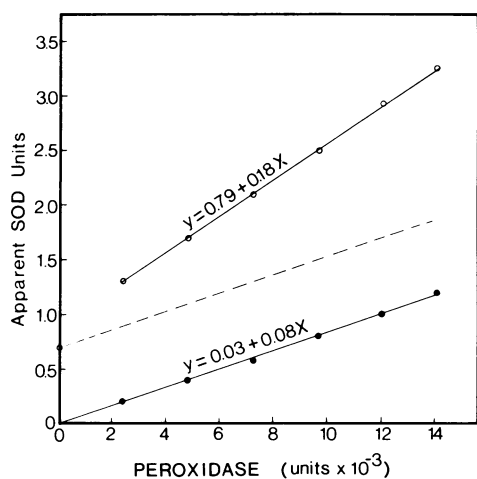


FIG. 5. Superoxide dismutase-like activity of HRP. The photochemical assay system for SOD was used. Reaction mixtures contained the indicated amounts of peroxidase (●—●), or the indicated amounts of peroxidase plus 0.7 unit of purified pea SOD (○—○). The synergistic effect of SOD on the SOD-like activity of peroxidase is apparent by the difference in the slope of the predicted (○---) and observed (○—○) lines for 0.7 unit of SOD.

accompanied by any loss of SOD activity in the corn and oat extracts. Some loss of SOD activity was observed in the case of the pea extract and may not be due to the inactivation of peroxidase, since pea SOD seems to be less heat-stable than corn and oat enzyme. After heating for 20 min, corn, oat, and pea extracts retained 100, 30, and 20% of the SOD activity, respectively. The retained activity was cyanide-sensitive and nondialyzable suggesting that it is due to enzymic reactions. It may be concluded from these results that interference by peroxidase is negligible, and corrections in Tables I and II need not be made.

The heat stability of SOD, which was observed when the crude extracts were heated, was not observed when purified enzyme was heated. Corn and pea SOD partially purified with  $(\text{NH}_4)_2\text{SO}_4$  and acetone fractionation were completely inactivated by heating at 75 C for 2 to 3 min. Addition of corn crude extract to the purified corn and pea enzyme before heating provided partial protection. These results may suggest that impurities in the crude extracts, and especially in the corn crude extract, protect SOD from heat inactivation.

In another experiment, purified pea enzyme was mixed with crude extracts at various proportions and assayed. The SOD activity of the mixtures was equal to the sum of the activities, when the photochemical assay system was used; but, it was less than the sum of the activities, when the xanthine/xanthine oxidase assay system (17) was used. This observation provides evidence that the photochemical assay system is more reliable than the xanthine/xanthine oxidase assay system for the determination of SOD activity in crude extracts.

The possibility that some of the SOD bands may be due to peroxidase was examined (Fig. 6). Two of the 10 SOD bands obtained from the three species, namely SOD 2 of corn and SOD 10 of oats, coincided with peroxidase bands, indicating that these two bands may be artifacts due to peroxidase.

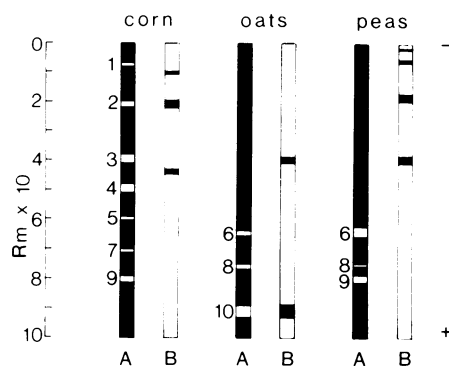


FIG. 6. Electrophoretic comparison of superoxide dismutases with peroxidases. Crude extracts from shoots of 10-day-old seedlings were applied on gels. For each species, one gel was stained for SOD (A), and one gel for peroxidase (B). Superoxide dismutase bands are numbered in order of increasing relative mobility as in Figures 2, 3, and 4.

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