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Superoxide Dismutases

II. PURIFICATION AND QUANTITATIVE RELATIONSHIP WITH WATER-SOLUBLE PROTEIN IN SEEDLINGS[1, 2]

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ABSTRACT

Superoxide dismutase was purified from pea (*Pisum sativum* L., cv. Wando) seeds and corn (*Zea mays* L., cv. Michigan 500) seedlings. The purified pea enzyme eluting as a single peak from gel exclusion chromatography columns contained the three electrophoretically distinct bands of superoxide dismutase characterizing the crude extract. The purified corn enzyme eluted as the same peak as the pea enzyme, and contained five of the seven active bands found in the crude extract. The similar molecular weights and the cyanide sensitivities of these bands indicated that they are probably isozymes of a cupro-zinc superoxide dismutase. One of the remaining corn bands was shown to be a peroxidase.

Superoxide dismutase accounted for 1.6 to 2.4% of the water-soluble protein in seedlings of corn, peas, and oats (*Avena sativa* L., cv. Au Sable). The superoxide dismutase activity per plant and per milligram water-soluble protein considerably increased during germination of oats and during greening and hook opening of peas.

In a previous study, considerable amounts of SOD[3] were found to be present in roots, shoots, seeds, and seed parts of oats, corn, and peas (4). Electrophoresis indicated multiple forms of the enzyme. Significant differences in quantity and forms of the enzyme were observed between species and between organs within a species. The objective of this study was to substantiate further the occurrence of the enzyme in higher plants and to examine the observed differences between species. For this purpose the enzyme was purified. Changes of SOD activity during seedling growth were also studied.

MATERIALS AND METHODS

Enzyme Purification. Unless otherwise stated, all operations were performed at 0 to 4 C. Dry pea seeds (*Pisum sativum* L., cv. Wando) were soaked in distilled H_2O for about 15 hr. The resulting 1650 g wet weight was crushed with an electric mortar and pestle and homogenized with 1 liter of 0.1 M K_2HPO_4 in a Waring Blendor. The resulting pH was 7.5. After stirring, the slurry was filtered/squeezed through six layers of cheesecloth. The filtrate was centrifuged twice at 13000*g* for 30 min in a Sorvall RC2-B refrigerated centrifuge.

The supernatant was subjected to the Tsuchihashi (chloroform-ethanol) treatment essentially as described by Weisiger and Fridovich (10). None of the pea SOD enzymes is inactivated by this treatment. The supernatant was mixed with 0.25 volume of ethanol and 0.15 volume of chloroform and stirred for 15 min. It was then clarified by centrifugation at 13000*g* for 15 min. Chloroform that was separated out during centrifugation was removed by suction. The supernatant was decanted, solid K_2HPO_4 (20 g/l) was added, and the two phases were separated after 30 min. The less dense, ethanol-rich phase was collected, chilled, and centrifuged at −15 C. Additional chloroform separating out during centrifugation was removed by suction, and the ethanolic phase was decanted.

Chilled acetone (0.5 volume) was added to the ethanolic phase while stirring. The precipitate was removed by centrifugation. Additional acetone (1.5 volume) was added to the supernatant, and the second precipitate was collected and redissolved in a minimal volume of 0.05 M K-phosphate (pH 7.8). Solid $(NH_4)_2SO_4$ was added to the supernatant to bring it to 70% saturation. After 1 hr, the second precipitate was collected, and resuspended in and dialyzed against 0.1 M KCl, 5 mM K-phosphate, and 10 μM EDTA (pH 7.8).

The enzyme was further purified by gel exclusion chromatography. It was first applied on a Sephadex G-100 column (2 × 90 cm) equilibrated with the dialysis buffer. The void volume of the column was 74.5 ml and the flow rate 0.2 ml/min. This column was calibrated with proteins of known mol wt (9). Fractions with a specific activity greater than 300 units/mg protein were pooled and salted out from 70% saturated $(NH_4)_2SO_4$ solution. The precipitate was collected by centrifugation, dissolved in a small volume of distilled H_2O, and dialyzed against the eluting buffer. It was then applied on a column (1 × 60 cm) of Bio-Gel P-30 equilibrated with the same buffer. Some impurities of higher mol wt were removed by this column. Fractions whose specific activity exceeded 700 units/mg were pooled and concentrated as above. The enzyme was rechromatographed on the same Bio-Gel P-30 column. Impurities of slightly lower mol wt were partially separated from the enzyme. Only the two fractions of maximum specific activity (around 2000 units/mg) were pooled this time. Additional impurities were removed by fractionation with chilled acetone. The most active fraction was obtained between 1.5 and 2 volumes of acetone. The precipitate from the last fraction was redissolved in 0.05 M K-phosphate (pH 7.8).

Enzyme from corn (*Zea mays* L., cv. Michigan 500) seedlings was partially purified. The seeds were treated with 0.3% (w/v) captan 80W for 5 min and germinated for 7 days on moist paper towels in the dark at room temperature. The seed remnants were removed, and the seedlings (182 g) were rinsed with distilled H_2O and cut into 1-cm sections with a stainless steel razor blade. The tissue was homogenized with 400 ml of 0.1 M K-phosphate and 0.1 mM EDTA (pH 7.8) in a Waring Blendor. After 1 hr in the cold and occasional stirring, the homogenate was filtered/squeezed through four layers of cheesecloth. The filtrate was centrifuged twice at 13000*g* for 20 min. This supernatant was

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[3] Abbreviations: SOD: superoxide dismutase (EC 1.15.1.1); NBT: *p*-nitro blue tetrazolium chloride.

not subjected to the Tsuchihashi treatment (10), since it has been shown that this treatment inactivates one of the corn enzymes (4). The enzyme was successively purified by acetone fractionation (0.75–2 volumes), $(NH_4)_2SO_4$ fractionation (45–95% saturation) and chromatography on Sephadex G-100 in a manner similar to the pea enzyme.

Protein concentration was determined throughout according to Lowry *et al.* (7), using BSA as a standard. Enzyme assays and electrophoresis were performed as previously described (4).

Changes in SOD Activity of Seedlings. The changes in specific activity during greening were studied with excised oat (*Avena sativa* L., cv. Au Sable) and pea plumules. Seeds, treated with 0.3% (w/v) captan 80W for 5 min, were soaked in distilled H_2O for 10 hr and planted 2-cm deep in Styrofoam pots (10 × 14.5 cm) containing vermiculite. Seedlings were grown in a growth chamber in complete darkness at 25 C for 7 days. Plumules were excised by cross-secting with a stainless steel razor blade above the first node from the apex. Uniform plumules were transferred into 9-cm Petri dishes (10 plumules/dish) containing 10 ml of 1% sucrose solution. Three replicate dishes for each treatment were prepared. Half of the dishes were placed in a growth chamber with all lights off. The other half were placed in another growth chamber with only the fluorescent lights on (10 and 7 $\mu w/cm^2$ blue and red light, respectively). The temperature in both chambers was maintained at 25 C. At various time intervals, Petri dishes were removed from the chambers, the plumules were rinsed with distilled H_2O, blotted, and weighed. Extracts were prepared and the assays were performed immediately. All operations with etiolated plumules were conducted under dim green safelights.

The changes of specific activity during germination were studied with oat and pea seedlings. Seeds were treated as in the previous test and placed on two layers of filter paper in 9-cm Petri dishes (15 seeds/dish). Five ml of distilled H_2O was added to each dish, and the dishes were transferred into a dark growth chamber at 25 C. After 2 days, 10 uniform seedlings/dish were harvested daily at a fixed time. The roots and shoots were separated, rinsed, and weighed prior to extraction and assaying. The experiment was repeated three times.

RESULTS AND DISCUSSION

Enzyme Purification. Tsuchihashi treatment (10) of the pea extract resulted in a 5-fold purification and insignificant loss of activity, which is in agreement with earlier observation that pea SOD is resistant to this treatment (Table I). Subsequent acetone and $(NH_4)_2SO_4$ fractionation gave a 20-fold purification of the pea enzyme with good recovery. Purification of the corn enzyme with the Tsuchihashi treatment was not attempted due to the elimination of one of the SOD bands observed on gels. Only part of the enzyme could be precipitated from crude corn extract, and this explains the poor recovery after acetone fractionation. Fractionation of the crude corn extract with $(NH_4)_2SO_4$ also resulted in a low recovery. The crude corn SOD was also found to be heat-stable (4). Heat stability and resistance to precipitation may be related.

Table I. Purification of SOD from 500 g of Pea Seeds and 182 g of Corn Seedlings

Purification Step	Volume	Total Protein	Total Activity	Specific Activity	Yield	Purification
	ml	mg	units	units/mg	%	-fold
				Pea Seeds		
Crude Extract	1580	55774	780678	14.0	...	...
Ethanolic phase[1]	1543	10492	761316	72.6	97.5	5.2
Acetone fraction	401	4210	701750	166.7	89.9	11.9
$(NH_4)_2SO_4$ fraction	15	1842	528948	287.2	67.8	20.5
Sephadex G-100	637	567	319297	563.1	40.9	40.2
Biogel P-30 (1st)	88	231	235765	1020.6	30.2	72.9
Biogel P-30 (2nd)	12	38	76506	2002.8	9.8	143.1
Acetone fraction	5	29	59331	2039.0	7.6	145.6
				Corn Seedlings		
Crude extract	404	2513	30102	12	...	...
Acetone fraction	121	231	14109	61	46.9	5.1
$(NH_4)_2SO_4$	26	60	13013	216	43.2	18.0
Sephadex G-100	208	18	9482	539	31.5	44.9

[1]Separated out after the crude extract was treated with a chloroform-ethanol mixture (Tsuchihashi treatment).

Chromatography on a Sephadex G-100 column resolved the pea enzyme into a major and a minor peak (Fig. 1). Addition of mercaptoethanol to the eluting buffer converted the minor peak to the major one. The pooled fractions of the major peak, after they were concentrated and dialyzed, were rechromatographed twice on a Bio-Gel P-30 column. The enzyme eluted as a single peak from this column (Fig. 2). Enzyme from both the major Sephadex peak and the single Bio-Gel peak were compared with freshly prepared crude enzyme by electrophoresis. All three enzyme preparations gave banding pattern on gels identical to that previously described for pea SOD (4).

The corn enzyme eluted from the Sephadex column also as a major and a minor peak (Fig. 3). Enzyme from each peak was compared with freshly prepared crude enzyme by electrophoresis. The banding pattern previously described for corn SOD (4) was again obtained with the crude enzyme. The enzyme from the major Sephadex peak gave this banding also, except that SOD 2 and 5 were not present. The enzyme from the minor Sephadex peak gave three new faint bands which were not similar to any from the crude enzyme.

The following conclusions may be made from the chromatographic and electrophoretic behavior of the enzyme. The minor peaks eluted from the Sephadex column represent a small portion of the enzyme which was altered during purification. This is supported by the observation that electrophoresis indicates that these proteins are not present in the crude extracts. Weisiger and Fridovich (10) reported that chicken liver enzyme elutes also as a minor and a major peak from Sephadex, and that mercaptoethanol converts the former to the latter. They also have shown that the minor peak represents a polymeric form of the enzyme, and that aging in the cold promoted polymerization. During the

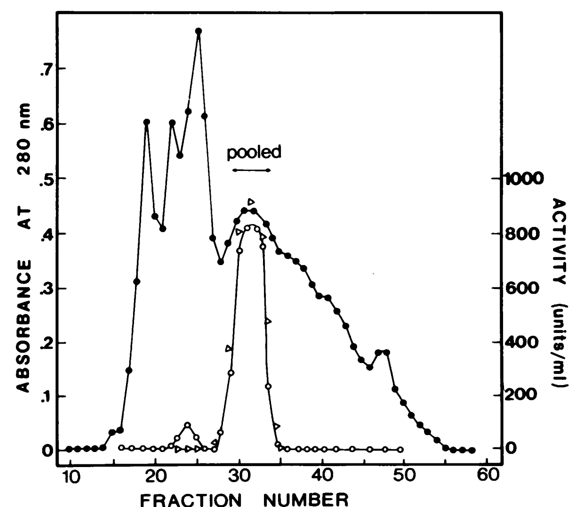

FIG. 1. Chromatography of pea SOD on Sephadex G-100. The 35 to 70% $(NH_4)_2SO_4$ precipitate was redissolved in and dialyzed against 0.1 M KCl, 5 mM K-phosphate, and 10 mM EDTA (pH 7.8). One ml of this solution was applied on a column (2 × 90 cm) equilibrated with the same buffer. Fractions of 5 ml were collected and assayed for SOD activity (○——○) and absorbance at 280 nm (●——●). The enzyme eluted as a major and a minor peak. When 1% mercaptoethanol was included in the eluting buffer, and an identical sample run on the same column, the minor peak was eliminated (△——△).

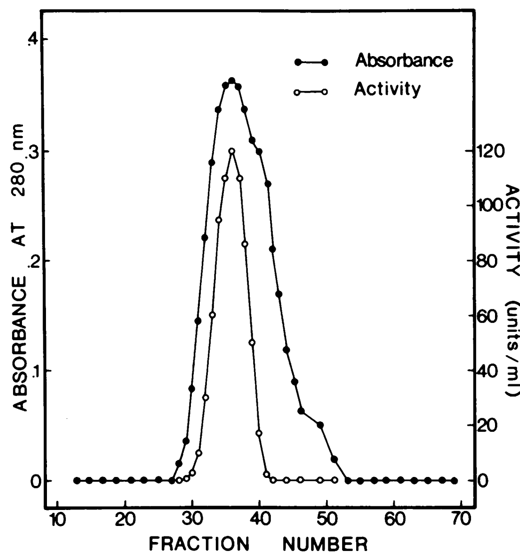

FIG. 2. Chromatography of pea SOD on Bio-Gel P-30 (2nd). The column (1 × 60 cm) was equilibrated and the enzyme eluted with the same buffer described in Figure 1. The enzyme collected from a first run on this column was concentrated to 0.5 ml, dialyzed, and rechromatographed.

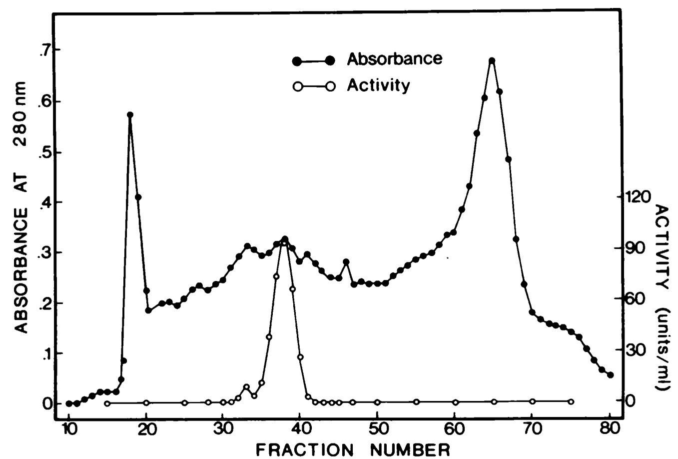

FIG. 3. Chromatography of corn SOD on Sephadex G-100. The column and buffer were the same as described in Figure 1. The 45 to 95% $(NH_4)_2SO_4$ precipitate was redissolved in and dialyzed against the buffer before loading the column. Fractions are 4 ml each.

course of this study, storage of pea or corn enzyme resulted in additional faint bands on gels. This indicates that the plant enzyme polymerizes similarly to the chicken enzyme. The polymerization apparently does not inactivate the enzyme. The major peaks eluted from the Sephadex column represent the bulk of the enzyme which did not undergo any alteration during purification.

The enzyme from each species eluting as the major peak from Sephadex is apparently homogeneous with regard to mol wt and heterogeneous with regard to electrophoretic properties. This supports the view that the SOD bands correspond to isozymes of SOD. The major peak obtained with pea enzyme contained all three SOD bands found in the crude extract. These bands could be eliminated with cyanide, indicating they are due to isozymes of cupro-zinc SOD. The major peak obtained with corn enzyme contained five out of the seven SOD bands found in the crude extract. All of these bands could be eliminated with cyanide and, thus, are isozymes of cupro-zinc SOD. Pea and corn cupro-zinc enzyme have the same mol wt (approximately 30,000) as indicated by similar elution volumes from the Sephadex columns (Figs. 1 and 3).

The two SOD bands of corn that were not present in the major peak were SOD 2 and 5. The SOD 2 band was a cyanide-sensitive protein which might be an artifact due to peroxidase (4). The SOD 5 band was a cyanide-resistant, chloroform-ethanol-sensitive protein, which may be a manganese-containing SOD. The manganese enzymes have a considerably higher mol wt than the cupro-zinc enzymes (3). Therefore, SOD 5 may have eluted from the Sephadex column as a separate peak and diluted to such an extent that it could not be detected.

The enzyme from oats was not purified. We showed (4), however, that one of the three SOD bands of oats may be an artifact due to peroxidase. The other two bands had the same relative mobility on gels as two of the pea bands and, therefore, they may also correspond to isozymes of cupro-zinc SOD.

Seedling Enzyme as Percentage of the Water-soluble Protein. The specific activity of the pea SOD was found to be 14 and 2039 photochemical units/mg protein for the crude and the purified enzyme, respectively (Table I). The enzyme from pea seeds was also purified by Sawada *et al.* (9). They determined a specific activity for the pure enzyme equivalent to 2112 photochemical units/mg, which is similar to the specific activity found in this study. Their specific activity for the crude enzyme was 3.3, considerably lower than determined in this study. Sawada *et al.* (9) used the xanthine/xanthine oxidase assay system (8) for their assays. Evidence was obtained in the course of a previous study that impurities in crude extracts depress the enzyme activity when determined by this assay system (4). This may explain the low specific activity of the crude enzyme as determined by Sawada *et al.* (9).

The purified pea enzyme was tested for purity by electrophoresis. One weak band not corresponding to any SOD-active protein was localized on the gels. The actual specific activity of the enzyme, therefore, is expected to be somewhat higher than the above value. Asada *et al.* (2) have purified spinach leaf SOD to a crystalline state. The specific activity of this enzyme was equivalent to 3076 photochemical units/mg. Their method of protein determination (absorption at 258 nm) was different from the Lowry procedure used in this study and by Sawada *et al.* (9), thus, a direct comparison of the values may not be relevant. It is reasonable to assume that the specific activity of SOD in higher plant species is within the range of 2039 to 3076.

The specific activity of the crude enzyme in roots and shoots of seedlings has already been determined (4). These data were used here to estimate the percentage of water-soluble protein in seedlings accounted for by SOD, assuming specific activities for the pure enzyme in the range 2039 to 3076. Averaged over shoots and roots of the three species studied, SOD accounts for a minimum of 1.6% and a maximum of 2.4% of the water-soluble protein in seedlings. It should be noted that these percentages of water-soluble protein accounted for by SOD refer to 10-day-old seedlings grown under the given conditions. The figures are not as expected from the purification data (Table I) obtained from pea seeds, apparently because pea seeds are much richer in water-soluble protein than seedlings.

Changes in SOD Activity of Seedlings. Evidence for the inducibility of SOD has been presented for bacteria (5, 6) and blue-green algae (1). In this study, SOD specific activity was shown to increase during greening of pea plumules and germination of oats.

Pea and oat plumules excised from seedlings grown in the dark were either kept in the dark or transferred to the light. After 48 hr, the SOD specific activity of the green plumules was compared with that of the etiolated ones (Table II). Green pea plumules had developed a specific activity which was approxi-

mately 40% higher than that in the etiolated pea plumules. Green and etiolated oat plumules had developed the same specific activity. A different growth response to light by the two species seems to be associated with these results. Over the 48-hr period, pea plumules grew faster in the light (hook opening, plumular expansion) than in the dark. Oat plumules grew at the same rate in the dark and light. On a fresh weight basis, green oat plumules accumulated more SOD than etiolated oat plumules; but they also accumulated proportionally more water-soluble protein and thus the specific activity did not increase. Associated with increased growth rate of pea plumules in the light is a decrease of water-soluble protein but not of SOD, resulting in an increase of the specific activity with greening.

Results from a kinetic experiment with pea plumules indicated that the increase of SOD preceded that of growth (Fig. 4). Although, on a per plumule basis, water-soluble protein decreased with time at the same rate both in the dark and light, SOD increased. The increase of SOD was faster in the light. Growth in the light was accelerated after a lag period of 12 hr. The rapid increase in SOD preceded the acceleration of growth.

Table II. Growth, Water-soluble Protein, and SOD Activity of Plumules.

Plumules were excised from 7-day-old seedlings grown in the dark, placed in petri dishes containing 1% sucrose, and kept at 25 C in the dark (E) or under cool white light (G) for 48 hr.

Species	Etiolated (E) or Green (G)	Fresh wt (mg/plumule)	SOD (units/ g fresh wt)	Protein (mg/ g fresh wt)	SOD (units/ mg protein)
Oats	E	36.5	712.0	12.2	59.3
Oats	G	36.8	992.7*	16.9*	58.7
Peas	E	39.6	883.7	23.9	37.4
Peas	G	67.0*	901.0	17.3*	52.5*

* F value for difference between the means within a species is significant at the 0.05 level.

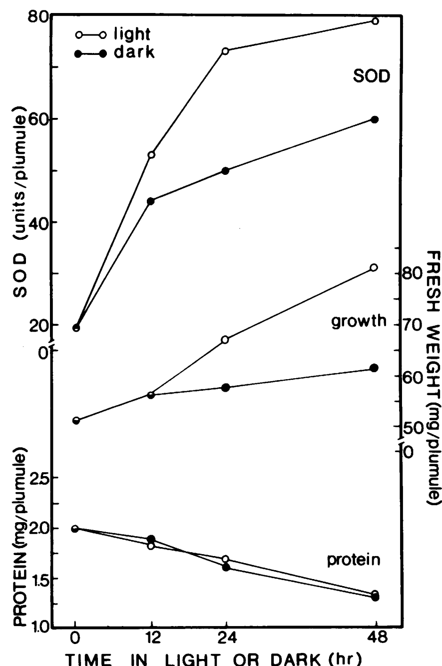

FIG. 4. Development of SOD activity with growth and water-soluble protein content of excised pea plumules. Plumules were excised from 7-day-old seedlings grown in the dark, placed in Petri dishes containing 1% sucrose solution, and kept in either dark or light conditions.

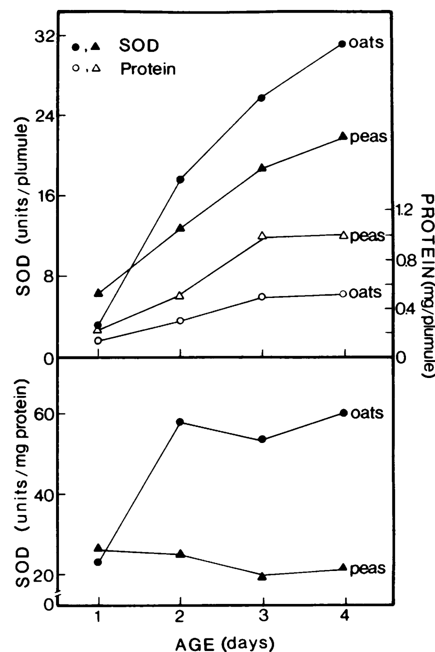

FIG. 5. Development of SOD activity during germination of oats and peas. Seeds were germinated in the dark, on moist filter paper in Petri dishes.

In these experiments, plumules were grown in the absence of any source of nitrogen. Conversion of water-soluble protein to insoluble protein may explain the decrease of the former.

The development of the SOD specific activity in oats and peas was also examined during the first days of germination (Fig. 5). On the 1st day, the specific activity was approximately the same in oats and peas. The specific activity in oats rapidly increased and remained at a high level. In peas, it remained at the initial low level. From previous experiments with 10-day-old seedlings, it was observed that the SOD specific activity was considerably lower in peas than in oats (4). The results from this experiment confirm the observation and indicate that the difference between the two species is established since early during germination.

Electrophoresis indicated that the increase of SOD activity during greening of peas and germination of oats is neither accompanied by any apparent shift in the relative amounts of the isozymes, nor the appearance of new isozymes.

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