

# Isoenzymes of Superoxide Dismutase in Nodules of *Phaseolus vulgaris* L., *Pisum sativum* L., and *Vigna unguiculata* (L.) Walp.<sup>1</sup>

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## ABSTRACT

The activity and isozymic composition of superoxide dismutase (SOD; EC 1.15.1.1) were determined in nodules of *Phaseolus vulgaris* L., *Pisum sativum* L., and *Vigna unguiculata* (L.) Walp. formed by *Rhizobium phaseoli* 3622, *R. leguminosarum* 3855, and *Bradyrhizobium* sp. BR7301, respectively. A Mn-SOD was present in *Rhizobium* and two in *Bradyrhizobium* and bacteroids. Nodule mitochondria from all three legume species had a single Mn-SOD with similar relative mobility, whereas the cytosol contained several CuZn-SODs: two in *Phaseolus* and *Pisum*, and four in *Vigna*. In the cytoplasm of *V. unguiculata* nodules, a Fe-containing SOD was also present, with an electrophoretic mobility between those of CuZn- and Mn-SODs, and an estimated molecular weight of 57,000. Total SOD activity of the soluble fraction of host cells, expressed on a nodule fresh weight basis, exceeded markedly that of bacteroids. Likewise, specific SOD activities of free-living bacteria were superior or equal to those of their symbiotic forms. Soluble extracts of bacteria and bacteroids did not show peroxidase activity (EC 1.11.1.7), but the nodule cell cytoplasm contained diverse peroxidase isozymes which were readily distinguishable from leghemoglobin components by electrophoresis. Data indicated that peroxidases and leghemoglobins did not significantly interfere with SOD localization on gels. Treatment with chloroform-ethanol scarcely affected the isozymic pattern of SODs and peroxidases, and had limited success in the removal of leghemoglobin.

Superoxide anion radical ( $O_2^-$ ) is the product of univalent  $O_2$  reduction, an unavoidable process linked to respiration. In legume nodules,  $O_2^-$  may also be formed during the oxidation of nitrogenase proteins, ferredoxins, flavodoxins, and Lbs<sup>3</sup> (for a review see ref. 4). Toxic effects of  $O_2^-$  are commonly attributed to its ability to generate the more powerful hydroxyl radical ( $\cdot OH$ ) via the metal-catalyzed Haber-Weiss reaction (12, 17). Though there is also some evidence that  $O_2^-$  *per se* can cause cellular damage (13), SODs (EC 1.15.1.1), in catalyzing the dismutation of  $O_2^-$  to  $H_2O_2$  and

$O_2$ , constitute a primary defense of cells against oxygen free radicals. Hydrogen peroxide, produced by SOD and other enzymic and nonenzymic reactions, is in turn removed by catalases and peroxidases (12, 27).

There are three types of SOD differing in their catalytic prosthetic metal (Cu plus Zn, Fe, or Mn). CuZn-SODs are mainly eucaryotic and have been localized into the cytosol, chloroplasts, mitochondrial intermembranous space, and glyoxysomes (27, 30). In contrast, the Fe- and Mn-SODs are characteristic of procaryotes, but the Mn-SODs also occur in mitochondria and in peroxisomes (31), and the Fe-SODs are present in the chloroplasts of some few species of higher plants (20, 28, 29, 33).

In a preliminary survey of legumes (3), we observed a varying number of CuZn-containing SODs in the nodule cytoplasm and of Mn-SODs in both bacteroids and cytoplasm. In the present work, we focus our investigation on three legume species and provide detailed information on SOD isoenzymes in free-living bacteria, bacteroids, and host cells. The occurrence of a Fe-containing SOD in the nonphotosynthetic tissue of a eucaryote is for the first time reported.

## MATERIALS AND METHODS

### Reagents

BSA and bovine CuZn-SOD were purchased from Boehringer Mannheim, and NBT was from Fluka. Cyt *c* (type III), lactalbumin, xanthine, and xanthine oxidase were from Sigma. All other chemicals were of analytical grade.

### Biological Material

Bacteria used were: *Rhizobium phaseoli* 3622, *R. leguminosarum* 3855, and *Bradyrhizobium* sp. BR7301. Legumes used were: *Phaseolus vulgaris* L. cv Canadian Wonder, *Pisum sativum* L. cv Lincoln, and *Vigna unguiculata* (L.) Walp.

### Bacterial and Plant Culture

Bacteria were grown at 28°C with orbital shaking in 100-mL Erlenmeyer flasks containing 40 mL of a yeast extract-mannitol-agar medium (pH 6.8) described elsewhere (3).

Surface-disinfected seeds were germinated in Petri dishes and immediately transferred to pots containing vermiculite that had previously been sterilized and washed with sterile N-

<sup>1</sup> Supported by grants PBT/15 from the CAICYT (Spain) (M. B., F. J. P.) and PB040401 from the DIGICYT (Spain) (L. M. S., L. A. R.).

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<sup>3</sup> Abbreviations: Lb, leghemoglobin; SOD, superoxide dismutase; NBT, nitroblue tetrazolium;  $R_m$ , relative mobility

free nutrient solution (26). Seedlings were then inoculated with 2 mL of a suspension containing  $10^{10}$  cells/mL of the appropriate bacteria. Plants were grown in a controlled-environment chamber set up to the following conditions: 25/15°C, 60/70% RH, and 16-h photoperiod ( $500 \mu\text{E m}^{-2} \text{s}^{-1}$  PAR). Plants were harvested at the following ages: *Phaseolus*, 33 d; *Pisum*, 30 d; and *Vigna*, 48 d. All of them were at the late vegetative stage.

### Sample Preparation

Bacterial cell-free extracts were prepared as indicated earlier (3), except that cells were disrupted by sonication with eight 1-min pulses at 1-min intervals (Branson B-12 sonifier, 90 W).

For isolation of the bacteroidal and plant fractions of nodule cells, 0.5 g nodules were washed and macerated gently using a mortar and pestle, with 10% (w/w) PVP and 6 mL of 0.1 M K-phosphate (pH 7.8) containing 0.1 mM  $\text{Na}_2\text{EDTA}$ . The slurry was squeezed through two layers of cheesecloth. After a first centrifugation at 650g for 5 min to eliminate nodule debris, bacteroids and plant soluble fraction ('cytoplasm') were collected as the 5,000g (for 10 min) pellet and 20,000g (for 15 min) supernatant, respectively. Bacteroids were washed twice and processed the same way as bacteria (3), except that bacteroids were resuspended in 3 mL instead of 5 mL buffer. A 3-mL aliquot of the plant fraction was treated with chloroform-ethanol (3) to assess the effect of organic solvents on SODs and peroxidases.

Mitochondria were isolated from 2.5 g (*Pisum*) or 5 g (*Phaseolus* and *Vigna*) nodules with 10 or 20 mL, respectively, of the extraction medium of Hanks *et al.* (18). The homogenate was filtered through six layers of cheesecloth and centrifuged at 650g for 5 min, 5,000g for 10 min, and 10,000g for 15 min. The 650g and 5,000g pellets were discarded and the 10,000g pellet was resuspended very gently, with a soft paint brush, in 3 mL of extraction buffer. Small aliquots of this suspension were sonicated, treated with 0.1% Triton X-100 (final concentration) to solubilize membranes, and cleared by centrifugation. The resulting supernatant was used for analysis of SOD isozymes.

Maceration of nodules and preparation of all samples were made at 0 to 2°C. Special care was taken when handling mitochondrial suspensions to minimize any chance of disruption by excessive shaking, pipetting or heating of samples.

### Enzyme Assays

Prior to the determination of SOD activity, samples were extensively dialyzed against 5 mM K-phosphate (pH 7.8) and 0.1 mM  $\text{Na}_2\text{EDTA}$  to eliminate low mol wt reductants that can interfere with the SOD activity assays. Total SOD activity was determined essentially by the procedure of McCord and Fridovich (22), based on the SOD ability to inhibit the reduction of ferricytochrome *c* by  $\text{O}_2^-$  generated by the xanthine/xanthine oxidase system. SOD activity was routinely assayed in the presence of 10  $\mu\text{M}$   $\text{CN}^-$  to suppress Cyt *c* oxidase without affecting CuZn-SOD (6). One unit of SOD activity was defined as the amount of enzyme required to inhibit the ferricytochrome *c* reduction by 50% at 25°C (22).

Peroxidase (EC 1.11.1.7) was measured by following the oxidation of *o*-dianisidine at 460 nm (14), and catalase (EC 1.11.1.6) was determined by the UV method described by Aebi (1). Cyt *c* oxidase (EC 1.9.3.1) and alanine dehydrogenase (EC 1.4.1.1) were determined according to Schnarrenberger *et al.* (32) and by a slightly modified procedure (3) of that of Reibach *et al.* (25), respectively. For the assay of catalase, Cyt *c* oxidase, and alanine dehydrogenase, samples were preincubated for 2 to 3 min with 0.05%, 0.25%, and 5% Triton X-100 (final concentrations), respectively. All enzyme activities were determined with a Beckman DU-7 spectrophotometer.

Proteins were determined by the method of Bradford (5), using a standard of BSA, and, when required, samples were treated with 0.02% Triton X-100 (final concentration). This concentration did not interfere with protein determination.

### Separation of SOD Isoenzymes and Lb Components

Samples (50–80  $\mu\text{L}$ ) were electrophoresed on 10% acrylamide disc gels at pH 8.9 under nondenaturing conditions, using the Tris-glycine system of Davis (10). Isozymes of SOD were visualized on gels by the method of inhibition of NBT reduction by  $\text{O}_2^-$  radicals generated photochemically (36). The three types of SOD were differentiated by performing the activity stains in gels previously incubated at 25°C for 45 min in 50 mM K-phosphate buffer (pH 7.8), containing 2 mM  $\text{CN}^-$ , 2 mM  $\text{H}_2\text{O}_2$  and/or 5 mM  $\text{H}_2\text{O}_2$ . CuZn-SODs are inhibited by  $\text{CN}^-$  and  $\text{H}_2\text{O}_2$ , Fe-SODs are resistant to  $\text{CN}^-$  but inactivated by  $\text{H}_2\text{O}_2$ , and Mn-SODs are resistant to both inhibitors (27).

For peroxidase localization, gels were incubated at 25°C for 1 h in a solution containing 0.2 M K-phosphate (pH 6.0), 0.33 mM *o*-dianisidine, and 3 mM  $\text{H}_2\text{O}_2$  (14).

The activity bands of SOD and peroxidase, and Lbs were recorded with a Vernon PHI-6 densitometer. Some of the Lb isoproteins (those from *Phaseolus* and *Vigna*) were also identified by their red color. The SOD isozymes and Lbs were quantitated from the areas under the peaks of the densitometer tracings.

### Mol Wt Determination

The mol wt of SODs was estimated by electrophoresis at different polyacrylamide gel concentrations (5–11%), as indicated by Hedrick and Smith (19). Lactalbumin (14,200), bovine CuZn-SOD (32,500), and BSA (monomer: 67,000; dimer: 134,000; trimer: 201,000) were used as standards and SODs were localized on gels by the photochemical method described above.

## RESULTS

Soluble extracts of *Bradyrhizobium*, bacteroids and nodules contain several electrophoretically distinct SODs (Table I; Fig. 1). This confirms earlier results, in which nodule extracts were stored in 25% glycerine at  $-30^\circ\text{C}$  for several days before electrophoresis (3), and indicates that multiple SODs were not an artifact of storage of samples. Furthermore, it does not seem likely that isozymes are artifacts derived from the extraction procedure in view of the similar number of isozymes

**Table I.** Isozymes of SOD in Free-Living and Symbiotic *Rhizobium-Bradyrhizobium* and in Nodules of Several Leguminous Plants

Isozymes were numbered in order of increasing  $R_m$ .  $R_m$ -values are means of three (bacteria, bacteroids) or six (nodule plant fraction) separate extracts. Percentages are means of three separate extracts. SE < 10%.

Symbiosis	Free-Living Bacteria			Bacteroids			Nodule Plant Fraction			
	Isozyme	$R_m$	%	Isozyme	$R_m$	%	Isozyme	$R_m$	%	% <sup>a</sup>
<i>P. vulgaris</i> / <i>R. phaseoli</i>	Mn-1	0.47	100	Mn-1	0.27	34	Mn-1	0.28	12	7 NS
				Mn-2	0.42	66	CuZn-1	0.49	68	81 NS
							CuZn-2	0.65	20	12 ** <sup>b</sup>
<i>P. sativum</i> / <i>R. leguminosarum</i>	Mn-1	0.36	100	Mn-1	0.33	39	Mn-1	0.31	21	17 NS
				Mn-2	0.41	61	Mn-2	0.39	25	19 NS
							CuZn-1	0.47	47	55 NS
							CuZn-2	0.55	7	8 NS
<i>V. unguiculata</i> / <i>Bradyrhizobium</i> sp.	Mn-1	0.12	27	Mn-1	0.14	56	Mn-1	0.18	3	6 NS
	Mn-2	0.15	73	Mn-2	0.26	44	Mn-2	0.32	11	5 **
							Fe-1	0.45	7	8 NS
							CuZn-1	0.53	48	53 NS
							CuZn-2	0.57	13	12 NS
							CuZn-3	0.62	6	5 NS
							CuZn-4	0.70	13	11 NS

<sup>a</sup> Relative proportions of isozymes after chloroform-ethanol treatment of extracts.

<sup>b</sup> \*\*, Significant at  $P < 0.01$  based on Duncan's multiple range test for comparisons of two means.

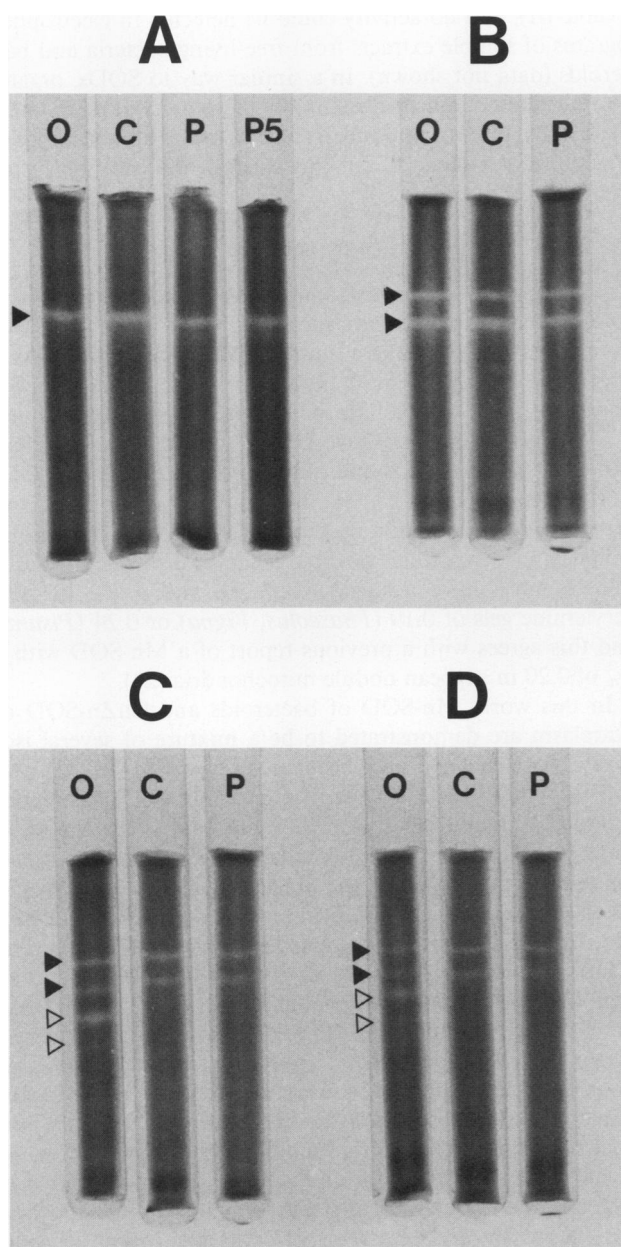
found in both cases (*cf.* Table I, ref. 3). Thus, one and two Mn-SODs were detected in *Rhizobium* and *Bradyrhizobium*, respectively; also, two Mn-containing SODs were observed in the bacteroid fraction of all legumes. There are, however, some differences with regard to the CuZn-SODs: two in *Phaseolus* and *Pisum* are reported here (Table I; Fig. 1), whereas three and one, respectively, were observed previously (3). The reasons for these variations may be the different acrylamide concentration used in this work (10% instead of 7.5%) and the fact that CuZn-SODs are much more labile than Mn- or Fe-SODs when stored in buffer for a few days, even at  $-70^{\circ}\text{C}$  (data not shown). No CuZn-containing SOD activity was found in the bacteroid fraction of any species.

Bacterial and plant Mn-SODs were compared tentatively by using  $R_m$ -values. Isolated mitochondria had a single SOD isozyme which, as judged by its insensitivity towards  $\text{CN}^-$  and  $\text{H}_2\text{O}_2$ , belongs to the class of Mn-SODs (Fig. 2). Mitochondrial Mn-containing SODs from *Phaseolus*, *Pisum*, and *Vigna* nodules exhibited  $R_m$ -values ( $\pm 0.01$ ,  $n = 3$ ) of 0.19, 0.24, and 0.19, respectively (Fig. 2). The assay of marker enzymes of mitochondria (Cyt *c* oxidase), peroxisomes (catalase), and bacteroids (alanine dehydrogenase) revealed that mitochondrial extracts were contaminated with peroxisomes, but not with bacteroids. The virtual absence of bacteroid contamination in our mitochondrial preparation was probably due to the introduction of a centrifugation at 5,000g which pelleted bacteroids prior to mitochondria sedimentation at 10,000g. Further purification of mitochondria in discontinuous sucrose gradients following the method of Hanks *et al.* (18) had only partial success since the peak of peroxidase somewhat overlapped that of mitochondria (data not shown). In any case, only a Mn-SOD isozyme was detected both in crude mitochondria and in density-gradient partially purified mitochondria. On the other hand, the peak of catalase activity, char-

acteristic of peroxisomes, coincided in the three legume species with a maximum of peroxidase activity (data not shown).

Only one Mn-SOD of bacteroids was originally present in the corresponding free-living bacteria: Mn-2 of *R. phaseoli* bacteroids and Mn-1 of bacteroids of *Bradyrhizobium* and probably of *R. leguminosarum* (Table I). Comparison of  $R_m$ -values of bacteroidal (Table I) and mitochondrial Mn-SODs (given above) showed no evidence of cross-contamination between both nodule fractions, and therefore it can be concluded that bacteroids do have two Mn-SODs instead of the only one found at the free-living stage. Conversely,  $R_m$ -values of Mn-SODs found in the soluble fraction of nodules indicate that at least part of these proteins probably originated from the disruption of bacteroids or mitochondria. Mn-1 from *Phaseolus*, Mn-1 and Mn-2 from *Pisum*, and Mn-2 from *Vigna* probably come from bacteroids broken during extraction; Mn-1 from *Vigna* has a  $R_m$  identical to the mitochondrial Mn-isozyme, but most likely represents a mixture of the mitochondrial and bacteroidal Mn-1 isozymes, which exhibit very close mobilities (Table I). It should be borne in mind that chloroform-ethanol treatment did not cause major changes in the isozymic pattern of the nodule soluble fraction and only CuZn-2 from *Phaseolus* and Mn-2 from *Vigna* differed significantly at  $P < 0.01$  (Table I).

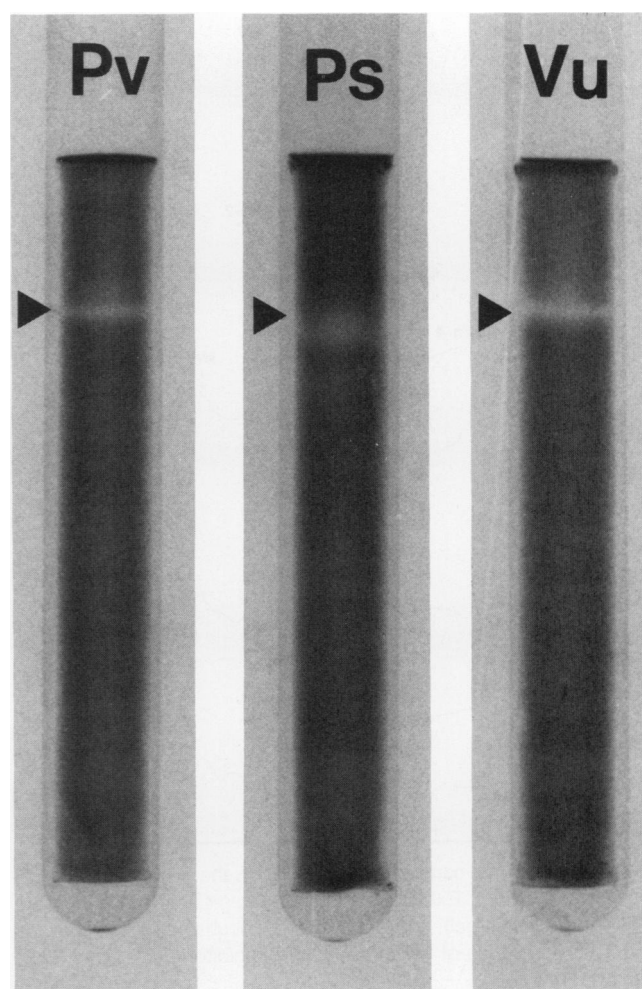
The finding of a Fe-SOD in the plant fraction of *V. unguiculata* nodules merits special attention. This isozyme displays a  $R_m$ -value intermediate of those of Mn- and CuZn-SODs, and represents about 8% of the total SOD activity in the host cells (Table I). The identification of the Fe-SOD was made on the grounds of differential inhibition: the protein was clearly resistant to  $\text{CN}^-$  but fully inactivated by  $\text{H}_2\text{O}_2$  (Fig. 3). As expected, CuZn-SODs were inhibited by  $\text{CN}^-$  and  $\text{H}_2\text{O}_2$ , and Mn-SODs were resistant to both inhibitors (Fig. 3). CuZn-1 in the soluble fraction of *Vigna* was responsible for about 50% of the total SOD activity (Table I). Hedrick and Smith



**Figure 1.** Localization of SOD isozymes on gels. Soluble extracts applied to gels were from (A) free-living *R. leguminosarum*, (B) *R. leguminosarum* bacteroids, (C) plant fraction of *Pisum* nodules, and (D) the same as C after treatment with chloroform-ethanol. Three or four aliquots of each extract were electrophoresed (10–40  $\mu\text{g}$  protein/gel), and gels were preincubated with buffer (O), or with buffer containing 2 mM  $\text{CN}^-$  (C), 2 mM  $\text{H}_2\text{O}_2$  (P), and/or 5 mM  $\text{H}_2\text{O}_2$  (P5). Gels were then stained for SOD activity as indicated in "Materials and Methods." ( $\triangleright$ ), CuZn-SODs; ( $\blacktriangleright$ ), Mn-SODs.

(19) analysis of isozymes Mn-2, Fe, and CuZn-1 from *Vigna* gave apparent mol wt of 75,000, 57,000, and 40,500, respectively (Fig. 4).

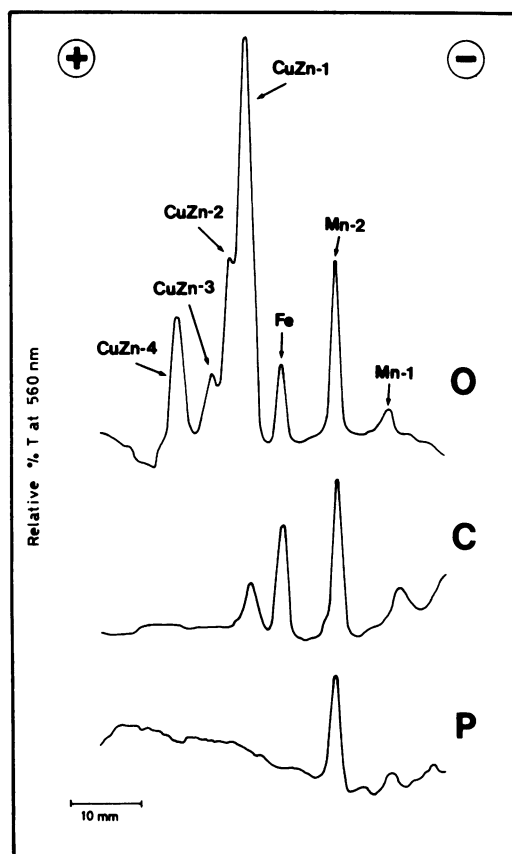
Despite the occurrence of two Mn-SODs in bacteroids, their total SOD activity was lower than, or at the most equal to, that of free-living bacteria (Table II). On a protein basis, only bacteroids from *Pisum* had similar SOD activity to that



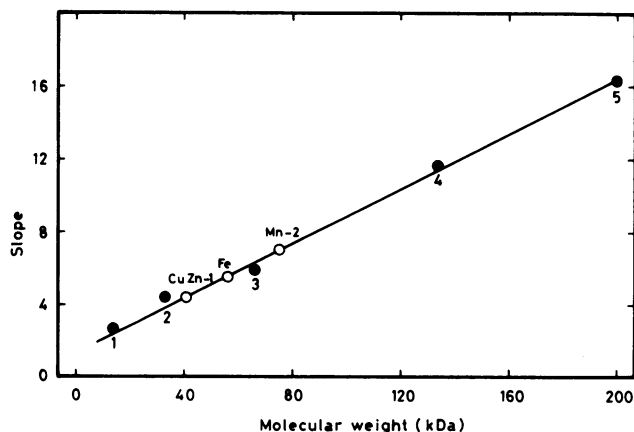
**Figure 2.** SODs in mitochondria from nodules of (Pv) *Phaseolus*, (Ps) *Pisum*, and (Vu) *Vigna*. Mitochondria were extracted, sonicated, and solubilized with Triton X-100 (see text for details). Extracts from each legume species were loaded on gels and run in triplicate. Then gels were incubated with buffer (control), 2 mM  $\text{CN}^-$  and 2 mM  $\text{H}_2\text{O}_2$ , and stained for SOD. Activity bands were resistant to both inhibitors, and therefore they correspond to Mn-SODs. Only control gels (no inhibitors) are shown here.

of the nodule soluble fraction. However, this fraction was treated with chloroform-ethanol which precipitates a large amount of proteins, and therefore the specific activities in the plant fraction probably represent an overestimation of the actual SOD activity. The results of the activity expressed per nodule fresh weight indicate that the whole capacity of bacteroids to scavenge  $\text{O}_2^-$  in nodules is considerably lower than that of host cells (Table II).

The plant fraction of nodules contained several electrophoretically distinct peroxidase bands (Table III). However, some of them are not true peroxidases, like Lbs, which exhibit pseudoperoxidase activity (9, 34). Lbs were identified by their red color and by densitometry.  $R_m$ -values ( $\pm 0.01$ ,  $n = 3-4$ ) for Lbs were as follows: 0.62, *Phaseolus* Lb I; 0.39, *Pisum* Lb I; 0.47, *Pisum* Lb II; 0.61, *Pisum* Lb IV; 0.73, *Pisum* Lb V; 0.62, *Vigna* Lb I. Lb III of *Pisum* was barely visible, and Lb II of *Vigna* migrated in front of the tracking dye. Thus,



**Figure 3.** Isozymic composition of SODs in the plant fraction of *V. unguiculata* nodules. Extracts were applied to gels and run in triplicate (30–40  $\mu$ g protein/gel), and gels were preincubated in the absence and in the presence of inhibitors. (O), no inhibitors; (C), 2 mM  $\text{CN}^-$ ; and (P) 2 mM  $\text{H}_2\text{O}_2$ .



**Figure 4.** Estimated mol wt of isozymes CuZn-SOD 1, Fe-SOD, and Mn-SOD 2 from the plant fraction of *V. unguiculata* nodules. Standards are: 1, lactalbumin; 2, bovine CuZn-SOD; 3, BSA, monomer; 4, BSA, dimer; and 5, BSA, trimer.

peroxidase 7 of *Phaseolus* corresponds to its single Lb and peroxidase 8 of *Vigna* to its Lb I. Peroxidases 4 to 6 of *Pisum* are also probably Lbs. In *Phaseolus* and *Vigna* Lbs accounted for approximately 20% of total peroxidase activity in nodules

(Table III), and no activity could be detected in electropherograms of soluble extracts from free-living bacteria and bacteroids (data not shown). In a similar way to SODs, organic solvents did not affect substantially the activity of peroxidases (Table III). Interestingly, nearly all *Phaseolus* Lb was removed by chloroform-ethanol, but this was not the case for *Pisum* and *Vigna* Lbs (Table III).

## DISCUSSION

Nitrogenase and Lbs are nodule proteins especially sensitive to activated oxygen species, such as  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  (4). However, there are in nodules a number of enzymes which have a protective function against the potential toxic effect of  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$ , and these include SODs, catalase, and peroxidases.

Two classes of SODs have been found in nodules so far: Mn-SODs in bacteroids and mitochondria, and CuZn-SODs in the cytosol (3, 11, 23, 24). In this paper we confirm and provide new information on the occurrence of such metalloenzymes in the nodules of three leguminous plants. Nodule mitochondria have a single Mn-SOD with a  $R_m$  in 10% acrylamide gels of 0.19 (*Phaseolus*, *Vigna*) or 0.24 (*Pisum*), and this agrees with a previous report of a Mn-SOD with a  $R_m$  of 0.20 in soybean nodule mitochondria (24).

In this work, Mn-SOD of bacteroids and CuZn-SOD of cytoplasm are demonstrated to be a mixture of several isozymes. Both enzymes have been already purified (11, 23), and two isozymes of the soybean nodule CuZn-SOD were observed (23). Dimitrijevic *et al.* (11) reported that a Mn-SOD and a Fe-SOD were present in free-living *R. phaseoli*, but that bacteroids only contained the Mn-isozyme (11). Stowers and Elkan (35) found a single SOD in several species of *Rhizobium* and *Bradyrhizobium* grown under aerobic conditions and, as judged by its behavior toward  $\text{CN}^-$ ,  $\text{H}_2\text{O}_2$ , and  $\text{N}_3^-$ , they identified the enzyme as a Fe-containing SOD. In a previous study (3), we preincubated the SOD-containing electropherograms with 2 mM  $\text{H}_2\text{O}_2$  for 45 min to discriminate Mn- and Fe-SODs; these conditions were assumed to be stringent enough to completely inactivate bacterial Fe-SODs, based on what occurs with their plant homologs. In the present work, we have used 5 mM  $\text{H}_2\text{O}_2$  for 45 min in extracts of free-living bacteria, and have confirmed our previous results showing that *Rhizobium* and *Bradyrhizobium*, grown in well-oxygenated complex media, possess one and two Mn-SODs, respectively, but not any Fe-SOD. Consequently, only a 2 mM  $\text{H}_2\text{O}_2$  concentration was employed for bacteroid and plant extracts.

Bacteroids contain two Mn-SODs, whereas free-living rhizobia have just one. In view of their dissimilar  $R_m$ -values compared to mitochondrial Mn-SODs, probably both isozymes belong to bacteroids and are not products of contamination. The presence of two Mn-SOD isozymes in bacteroids, contrary to the single one occurring in their respective free-living, non- $\text{N}_2$  fixing forms, supports the hypothesis that SODs protect the  $\text{N}_2$  fixation process (11, 23, 24). Nonetheless, total specific SOD activities of free-living bacteria are in general greater than those of bacteroids (Table II). A plausible explanation might be the different  $\text{O}_2$  environment of bacteria and bacteroids, which are exposed to almost anaerobic conditions in the infected region of nodules. No CuZn-SOD activity was detected in bacteroids, but a faint  $\text{CN}^-$ -sensitive

**Table II.** Total SOD Activity in Free-Living and Symbiotic Rhizobium-Bradyrhizobium and in Nodules of Several Leguminous Plants

Activities of the bacteroidal and plant fraction of nodules are expressed as units/mg protein or units/g nodule fresh weight (see "Materials and Methods" for unit definition). Data represent means of three separate extracts  $\pm$  1 SE.

Symbiosis	Free-Living Bacteria	Bacteroids		Nodule Plant Fraction	
	Units/mg protein	Units/mg protein	Units/g nodule	Units/mg protein	Units/g nodule
<i>P. vulgaris</i> / <i>R. phaseoli</i>	16.4 $\pm$ 0.1	7.1 $\pm$ 0.9	7.1 $\pm$ 0.1	17.7 $\pm$ 2.4	46.5 $\pm$ 1.5
<i>P. sativum</i> / <i>R. leguminosarum</i>	10.1 $\pm$ 1.1	13.2 $\pm$ 3.1	19.6 $\pm$ 0.5	10.2 $\pm$ 1.5	27.4 $\pm$ 2.6
<i>V. unguiculata</i> / <i>Bradyrhizobium</i> sp.	27.7 $\pm$ 5.4	9.5 $\pm$ 0.6	14.4 $\pm$ 0.5	16.9 $\pm$ 1.1	46.9 $\pm$ 1.8

**Table III.** Isozymes of Peroxidase in the Plant Fraction of Nodules from Several Leguminous Plants

Isozymes were numbered in order of increasing R<sub>m</sub>. Data are means of three (percentages) or six (R<sub>m</sub>-values) separate extracts. SE < 10%.

Plant Species	Isozyme	R <sub>m</sub>	%	% <sup>a</sup>	Comments
<i>P. vulgaris</i>	1	0.07	10	16 NS	
	2	0.13	7	10 NS	
	3	0.20	31	38 NS	
	4	0.30	4	8 NS	
	5	0.35	5	6 NS	
	6	0.47	25	20 NS	
	7	0.66	18	3 ** <sup>b</sup>	Lb I
<i>P. sativum</i>	1	0.04	25	38 NS	
	2	0.08	13	14 NS	
	3	0.22	14	14 NS	
	4	0.45	29	7 **	Possible Lb II
	5	0.63	5	7 NS	Possible Lb IV
	6	0.71	14	19 NS	Possible Lb V
<i>V. unguiculata</i>	1	0.11	21	29 **	
	2	0.19	24	23 NS	
	3	0.27	8	9 NS	
	4	0.37	14	14 NS	
	5	0.43	3	4 NS	
	6	0.53	9	6 **	
	7	0.56	4	1 NS	
	8	0.64	18	14 NS	Lb I

<sup>a</sup> Relative proportions of isozymes after chloroform-ethanol treatment of extracts. <sup>b</sup> \*\*, Significant at P < 0.01 based on Duncan's multiple range test for comparisons of two means.

activity, more cathodic than that of Mn-SODs, was previously found in the bacteroid fraction of *Phaseolus* nodules (3). Studies are now in progress to establish the conditions in which this putative CuZn-SOD activity, associated to the bacteroid fraction, can be observed.

A noteworthy result of this work is the finding of a Fe-SOD in the plant fraction of *V. unguiculata* nodules. This enzyme was absent in nodules of other legumes, including its related species *V. radiata* (3). Until now, the presence of Fe-SODs has been demonstrated only in the leaves of a few plant species (27, 33), and some of them have been isolated and characterized (20, 28, 29). In *Brassica campestris* and *Nuphar luteum*, Fe-SODs have been localized in the chloroplast stroma (28, 29). Like other Fe-SODs, the enzyme from *V. unguiculata* nodules is CN<sup>-</sup> resistant and H<sub>2</sub>O<sub>2</sub> sensitive, and has values of R<sub>m</sub> and mol wt intermediate between those of

CuZn- and Mn-SODs. The Fe-SOD activity accounted for about 8% of the total dismutase activity of nodule host cells, a value in the range (about 10%) of leaf Fe-SODs (Marvin Salin, personal communication). However, its mol wt (57,000), as estimated by the Hedrick and Smith (19) technique, is higher than those determined for other plant Fe-SODs using gel exclusion chromatography (41,000–46,000; refs. 20, 27, 28), but is very similar to that of *Euglena gracilis* Fe-SOD (57,500; ref. 21). As the CuZn- and Mn-SODs studied in this work, the Fe-SOD was not affected by chloroform-ethanol (Table I). These results are in conflict with the observation that Mn- and Fe-SODs from various sources are inactivated by organic solvents (15, 36).

Nodules also show the presence of several peroxidases. These isozymes can be visualized on gels using nonphysiological reductants, such as 4-chloro-1-naphthol (9) or *o*-dianisidine (this report), among other organic compounds. But other hemoproteins may also exhibit pseudoperoxidative activity when assayed with those substrates and, therefore, the possibility of interference should be considered. For instance, Lbs show high peroxidase activity with *o*-dianisidine in crude extracts (9) or in gels (this report). Other studies have demonstrated that pure Lb peroxidizes guaiacol (34). Nodules also contain ascorbate peroxidase (EC 1.11.1.11) and the soybean enzyme has been purified and characterized recently (9). It is abundant in nodules and can use *o*-dianisidine as electron donor, similar to Lbs and other nonspecific peroxidases (9). Therefore, it seems reasonable to think that some of the peroxidase activity bands being detected in polyacrylamide gels correspond to isozymes of ascorbate peroxidase.

Catalase is a typical enzyme of peroxisomes (12, 18) and may also exhibit pseudoperoxidative activity under certain conditions (12). Thus, the peroxidase activity detected in nodule peroxisomes could be attributed to either genuine peroxidase(s) or to catalase-mediated peroxidative activity. However, under the conditions of the peroxidase assay, the peroxidative activity of catalase is probably very small (16), and this is also evidenced by the fact that soluble extracts of bacteria and bacteroids do not show peroxidase activity in spite of having high levels of catalase activity (2, 8; this report).

On the other hand, peroxidase and catalase may give rise to 'peroxidative' artifacts when crude or electrophoresed extracts are assayed for SOD activity. For peroxidase, this is thought to occur by reoxidation of blue formazan by H<sub>2</sub>O<sub>2</sub> (15) or by inhibition of O<sub>2</sub><sup>-</sup>-dependent NBT reduction by O<sub>2</sub> formed by the decomposition of H<sub>2</sub>O<sub>2</sub>, in the case of catalase (7). Although some few SOD activity bands from the host

soluble fractions are coincident with peroxidases (*cf.* Tables I and III), several observations point out that they are true SODs: (a) 'peroxidative' artifacts require excess  $H_2O_2$ , and we have used a moderate  $H_2O_2$  concentration to preincubate gels (2 mM rather than 5 or 10 mM), which minimizes artifact production; (b) CuZn- and Fe-SOD activities were, as expected, fully abolished by preincubation of gels with  $H_2O_2$  (Figs. 1 and 3), whereas SOD-like activity of peroxidases and catalases should not be inhibited, but enhanced, under these conditions (7, 15); and (c) Mn-SOD activities ( $H_2O_2$ -resistant) of similar or equal  $R_m$ -values also occurred in bacteria and bacteroids (Table I), which do not contain peroxidase.

#### ACKNOWLEDGMENTS

The authors wish to thank Martha Daza for her valuable assistance in the molecular weight determination of superoxide dismutases. Thanks are also due to Dr. Marvin Salin (Mississippi State University) for helpful comments on the manuscript.

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