Plant Physiol. (1982) 70, 1321-1326
0032-0889/82/70/1321/06/$00.50/0

Characterization of a Manganese Superoxide Dismutase from the Higher Plant *Pisum sativum*[1]

Received for publication May 10, 1982 and in revised form July 2, 1982

FRANCISCA SEVILLA, JULIO LÓPEZ-GORGÉ, AND LUIS A. DEL RÍO
Unidad de Bioquímica Vegetal, Estación Experimental del Zaidín, C. S. I. C., Profesor Albareda 1, Granada, Spain

ABSTRACT

A manganese-containing superoxide dismutase (EC 1.15.1.1) was fully characterized from leaves of the higher plant *Pisum sativum* L., var. Lincoln. The amino acid composition determined for the enzyme was compared with that of a wide spectrum of superoxide dismutases and found to have a highest degree of homology with the mitochondrial manganese superoxide dismutases from rat liver and yeast. The enzyme showed an apparent pH optimum of 8.6 and at 25°C had a maximum stability at alkaline pH values. By kinetic competition experiments, the rate constant for the disproportionation of superoxide radicals by pea leaf manganese superoxide dismutase was found to be 1.61×10^9 molar^{-1} • second^{-1} at pH 7.8 and 25°C. The enzyme was not sensitive to NaCN or to H_2O_2, but was inhibited by N_3^-. The sulfhydryl reagent *p*-hydroxymercuribenzoate at 1 mM concentration produced a nearly complete inhibition of the manganese superoxide dismutase activity. The metal chelators *o*-phenanthroline, EDTA, and diethyldithiocarbamate all inhibited activity slightly in decreasing order of intensity. A comparative study between this higher plant manganese superoxide dismutase and other dismutases from different origins is presented.

Superoxide dismutase (EC 1.15.1.1) catalyzes the disproportionation of superoxide free radicals (O_2^-), common metabolic intermediates in a variety of biological oxidations, to molecular oxygen and H_2O_2 (29). It seems to play an important role in protecting cells against the indirect deleterious effects of these radicals (13). SODs[2] isolated from different sources belong to three classes containing either Fe, Mn, or Cu plus Zn as prosthetic groups.

In our laboratory, we are interested in the enzyme system SOD for its utility in studying the role and interactions between micronutrients Mn, Cu, Zn, and Fe in the plant cell (8). The isozyme pattern of SOD has been demonstrated to be useful for the estimation of the proportions of biologically active Mn, Cu, and Zn as well as to diagnose Mn deficiencies in *Pisum sativum* plants (8). Moreover, this enzyme system appears to be applicable to the differential diagnosis of Fe an Mn deficiencies (14). However, an obligate first step before undertaking studies on the role and mechanism of action of micronutrients at the subcellular level is to characterize fully those biocomplexes where micronutrients are incorporated.

In higher plants, several cuprozinc-SODs have been purified and characterized (1, 6, 39) but comparatively little information is available on the CN-insensitive Mn- and Fe-containing SODs. Two CN-resistant SOD have been partially purified from leaves of spinach (24) and kidney bean (20), and a similar enzyme has been partially characterized from maize kernels (4), although in no case was the metal content determined. The only exceptions are two Fe-SODs that have been isolated and characterized from mustard and water lily (35, 36).

Previously, we demonstrated the existence of three electrophoretically distinct SODs in pea leaves and tentatively identified one of them as Mn SOD based upon dependency of enzymic activity on the Mn nutrient levels supplied to the plant (8). This isozyme was subsequently purified to homogeneity and the presence of tightly bound Mn was confirmed and its Mn-protein stoichiometry established (40). The preliminary characterization of Mn SOD from *P. sativum* leaves showed that the enzyme contained 1 g atom Mn/mol enzyme, had a mol wt of 94,500 and was composed of four subunits of equal size with a mol wt of about 23,500 (41). This was the first report concerning the characterization of a Mn SOD from a higher plant.

In this paper, we report the amino acid composition, enzyme stability, rate constant for O_2^- dismutation, effect of potential inhibitors, and pH optimum of Mn SOD from *P. sativum* leaves. In the light of the results obtained, a comparative study between this higher plant Mn SOD and other SOD from different origins is presented.

MATERIALS AND METHODS

Pea plants (*Pisum sativum* L., var. Lincoln) were grown in the greenhouse in aerated full nutrient solutions under optimum conditions as described previously (9). The Mn SOD was purified to homogeneity from pea leaves by the procedure of Sevilla *et al.* (40).

SOD activity was assayed in terms of its ability to inhibit the superoxide-mediated reduction of ferricytochrome *c* (29) or of NBT (5) by XOD plus xanthine. In both cases, one unit of SOD activity was defined as that amount of enzyme which caused 50% inhibition of the initial rate of reduction of NBT or of ferricytochrome *c* at 25°C. Spectrophotometric measurements were performed at 25°C with a Perkin-Elmer 124 recording spectrophotometer. Protein concentrations were determined by the method of Lowry *et al.* (23).

Amino acid determinations of the purified enzyme were performed with a JEOL model JLC-6AH amino acid analyzer. Metalloprotein samples were hydrolyzed for 20 and 72 h at 110°C in 6 N HCl under vacuum. Half-cystine was determined as cysteic acid after oxidation with performic acid (32). Tryptophan was determined spectrophotometrically in 6 M guanidinium chloride according to the method of Edelhoch (10).

[1] This work was supported by a research grant from the Comisión Asesora de Investigación Científica y Técnica (Spain).

[2] Abbreviations: SOD, superoxide dismutase; Mn SOD, manganese-containing superoxide dismutase; Fe SOD, iron-containing superoxide dismutase; Cu-Zn SOD, copper-zinc-containing superoxide dismutase; NBT, nitro blue tetrazolium; DIECA, diethyldithiocarbamate; PHMB, *p*-hydroxymercuribenzoate; XOD, xanthine oxidase.

The second order rate constant for the dismutation of O_2^- by *P. sativum* leaf Mn SOD was determined by the method of Forman and Fridovich (11) based on the kinetic competition of O_2^- between SOD and ferricytochrome *c*.

The pH optimum of Mn SOD was determined by performing the ferricytochrome *c* assay (29) in 50 mM buffers at different pH values. The pH of the reaction mixtures was measured immediately after the enzyme addition whose final concentration varied between 0.25 and 1.6 μg/ml, depending upon the specific activity of the enzyme preparation used such that we always worked at a 50% inhibition value. Reaction controls were run for each pH value.

All inhibitors used were obtained from Merck except *p*-hydroxymercuribenzoate and DIECA which were purchased from Sigma. Commercially obtained inhibitors were not further purified. In all cases, the Mn SOD concentration in the activity assay mixture was fixed as described above. The potential inhibitors (NaCN, NaN_3, *o*-phenanthroline, EDTA, and DIECA) were prepared in 50 mM phosphate buffer, pH 7.8, and were added to the SOD assay mixture at different concentrations. However, PHMB and H_2O_2 were preincubated with the protein, and aliquots were subsequently taken for assay. Preincubations were carried out at 25°C using enzyme concentrations of 0.43 mg/ml and for 30 to 120 min and 1 h for each concentration of H_2O_2 and PHMB, respectively. In all experiments, SOD activity was determined by the xanthine-XOD/Cyt *c* method (29) except for DIECA, where the xanthine-XOD/NBT assay (5) was used to avoid interference by the metal chelator. SOD control reactions were carried out in the presence of each concentration of inhibitor, and reactions were initiated by adding an appropriate dilution of XOD so that the ΔOD/min, *i.e.* the generation rate of O_2^- radicals, was constant.

RESULTS

The amino acid composition of Mn SOD purified from leaves of *P. sativum* is shown in Table I. The enzyme has a high content of glutamic acid, leucine, alanine, and aspartic acid and contains a significant amount of half-cystine residues. Methionine was the less abundant amino acid.

Table I. *Amino Acid Composition of P. sativum Mn SOD*

Amino Acid	Residues/mol Enzyme[a]
Lysine	59
Histidine	31
Arginine	22
Aspartic acid	75
Threonine[b]	50
Serine[b]	55
Glutamic acid	81
Proline	39
Glycine	47
Alanine	79
Half-cystine[c]	14
Valine	62
Methionine	9
Isoleucine	53
Leucine	81
Tyrosine	36
Phenylalanine	30
Tryptophan[d]	32
Total residues	855

[a] Based upon a mol wt of 94,500.
[b] Determined by extrapolation to zero time.
[c] Determined as cysteic acid after performic acid oxidation.
[d] Determined spectrophotometrically in 6 M guanidine HCl (10).

The availability of higher amounts of purified Mn SOD permitted to calculate the molar extinction coefficient (ε) of the enzyme at 280 nm using higher protein concentrations, and to re-evaluate our preliminary results (41) with the calculation of a coefficient of $209 \times 10^3\ M^{-1} \times cm^{-1}$. This value is slightly higher than those described for Mn SOD from human liver (30), yeast (33), and bovine heart (27). This could be explained by the greater proportion in the Mn SOD from pea leaves of tryptophan residues, mainly responsible for the maximum absorption at 280 nm.

To determine the degree of relatedness between the *P. sativum* Mn SOD and SOD from different origins, a least-squares difference analysis of their amino acid compositions was performed (26). The calculated $S\Delta Q$ values are the sums of the squares of the difference between the mole percent of individual amino acid residues in the two proteins compared. The values obtained are an indication of relative sequence homology between proteins; the lower the number, the smaller the difference in amino acid content and, therefore, a greater relatedness. Table II gives the $S\Delta Q$ values calculated for 18 amino acids of 17 different SODs.

The effect of pH in the range of 4.1 to 10.6 on pure Mn SOD was next examined. Mn SOD at a concentration of 30 μg/ml was incubated at 25°C at a given pH. Activity was measured at 5 and 24 h by the NBT method. The Mn SOD was rapidly inactivated at pH 4.1 but at pH 5.1 retained 62% of its activity after 24 h. At pH values in the range 8.0 to 10.6, the enzyme was nearly fully active for 24 h at 25°C, with more than 95% of activity remaining.

The enzyme is unstable at higher temperatures (50°C, 60°C, and 80°C) as it has been previously described (40) but at low temperatures was stable at pH 7.5. After 4 months at −20°C and 4°C, the enzyme retained 80% and 75% of the initial activity, respectively.

The apparent pH optimum of Mn SOD from pea leaves was determined by carrying out the enzymic reaction in different buffers in a pH range between 6.0 to 10.6. The generation of O_2^- radicals was maintained constant at each pH by varying the amount of XOD in order to obtain a constant ΔOD/min (control rate). Mn SOD activity showed a maximum value at a pH of about 8.6 (Fig. 1).

The effect of different concentrations of pure Mn SOD on the initial O_2^--mediated reduction of Cyt *c* was investigated (Fig. 2). By interpolation, it was calculated that a SOD concentration of 3.72×10^{-9} M produced a 50% inhibition in Cyt *c* reduction. On

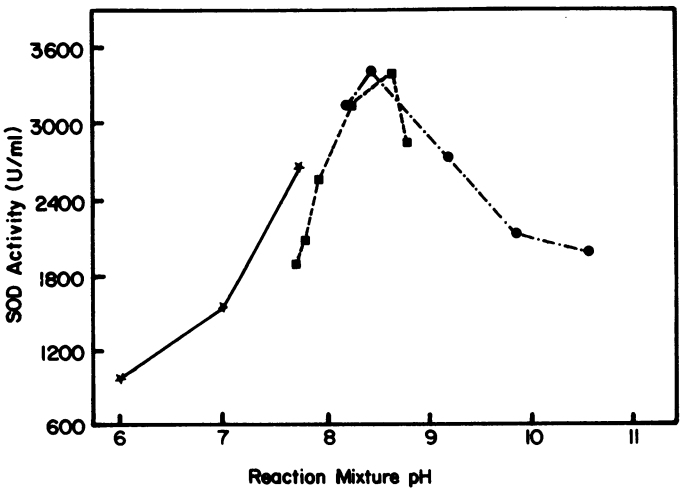

FIG. 1. Effect of pH on the enzymic activity of pea leaf Mn SOD. The enzyme reaction using the Cyt *c* method (29) was carried out in the following buffers at a 50 mM concentration: (★——★), phosphate; (▲ - -■), Tris-HCl; (●-·-●), glycine-NaOH. The pH of the reaction mixtures was determined immediately after the addition of the enzyme (zero time). Reaction controls were run for each pH value.

Table II. *Comparison of SΔQ Values of Different Superoxide Dismutases and the Pea Leaf Mn-Enzyme*

Values for $S\Delta Q$ were calculated from the amino acid composition of SOD reported in Table I and in previous literature using the following equation (26):

$$S\Delta Q \equiv \sum_j (X_{i,j} - X_{k,j})^2$$

where i and k indicate the enzymes which were compared and X_j is the mole percent content of a given amino acid of type j. In calculating $S\Delta Q$ values, 18 amino acids were used. Numbers in parentheses refer to literature from which data were taken.

		(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)	(j)	(k)	(l)	(m)	(n)	(o)	(p)
(a)	Pisum Mn-SOD																
(b)	Escherichia Mn-SOD (42)	54															
(c)	Thermus th. Mn-SOD (38)	64	68														
(d)	Thermus aq. Mn-SOD (37)	65	63	23													
(e)	Rhodopseudomonas Mn-SOD (25)	98	41	125	113												
(f)	Porphyridium Mn-SOD (31)	67	60	90	87	74											
(g)	Saccharomyces Mn-SOD (33)	49	28	47	48	71	73										
(h)	Rat liver Mn-SOD (34)	29	53	39	54	113	63	27									
(i)	Human liver Mn-SOD (30)	64	49	36	46	105	74	18	19								
(j)	Pea seeds Cu,Zn-SOD (39)	286	275	286	297	269	124	232	207	209							
(k)	Spinach leaves Cu,Zn-SOD (1)	209	197	183	209	218	78	175	150	153	54						
(l)	Chromatium Fe-SOD (17)	44	54	104	88	45	55	58	67	83	250	189					
(m)	Escherichia Fe-SOD (43)	66	34	88	69	28	28	56	78	79	208	148	38				
(n)	Plectonema Fe-SOD (3)	88	26	78	83	19	64	56	88	79	263	174	52	24			
(o)	Euglena Fe-SOD I (18)	99	102	62	52	102	67	88	92	84	237	165	89	55	74		
(p)	Brassica Fe-SOD (35)	36	34	53	60	63	32	34	21	35	191	123	32	31	40	58	
(q)	Nuphar Fe-SOD (36)	105	63	100	94	75	80	73	64	60	225	194	71	74	62	86	38

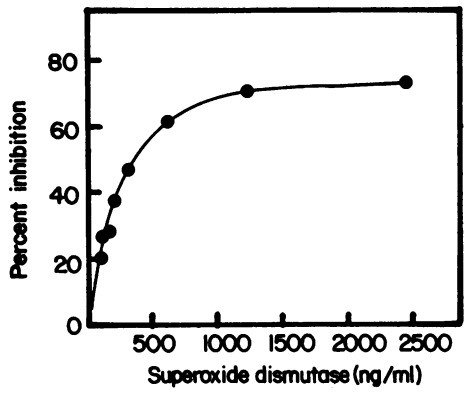

FIG. 2. Effect of different concentrations of pea Mn SOD on the enzymic activity. The SOD activity was assayed by the Cyt *c* method (29). Superoxide radicals were generated at 25°C by the action of XOD on 5 × 10^{-5} M xanthine in 0.05 M K-phosphate, 10^{-4} M EDTA, pH 7.8. Cyt *c* was present at 1 × 10^{-5} M and the enzyme activity was expressed as percentage of inhibition of Cyt *c* reduction by Mn SOD.

this basis and by using a kinetic competition method (11), the apparent second order rate constant for the enzymic disproportionation of O_2^- radicals by *P. sativum* Mn SOD was found to be 1.61 × 10^9 M^{-1} s^{-1} at pH 7.8 and 25°C.

Table III. *Effect of Enzyme Incubation with p-Hydroxymercuribenzoate on the Manganese Superoxide Dismutase Activity*

The incubation was carried out at 25°C for 60 min in a buffer containing 50 mM K-phosphate, pH 7.8.

PHMB	Inhibition
mM	%
0.1	0
1.0	92.4

Results on the effect of several compounds on Mn SOD activity showed that neither NaCN (0.1 mM to 2.0 mM) nor H_2O_2 (0.1 mM to 1.0 mM) produced any inhibition throughout the whole range of concentrations used, as could be anticipated for a SOD containing Mn as the metal prosthetic group (3). On the contrary, the incubation with 1 mM PHMB brought about a nearly complete inhibition of the Mn SOD activity, as shown in Table III. Table IV presents the effect of different concentrations of the Cu chelator DIECA on Mn SOD activity. The effects of other inhibitors: N_3^-, EDTA, and *o*-phenanthroline on Mn SOD activity are shown in Figures 3, 4, and 5, respectively. In all cases, controls were routinely performed to ensure that the inhibitory compounds added along with the enzyme did not interfere with the SOD assay *per se*.

Table IV. *Effect of Different Concentrations of DIECA on the Superoxide Dismutase Activity of Pure Mn Superoxide Dismutase*

DIECA was added to the SOD reaction mixture and the enzymatic activity was determined by the Cyt *c* method (29).

DIECA	Inhibition
mM	%
0.25	0
0.50	5.2
1.00	13.2
5.00	24.4
10.00	23.6

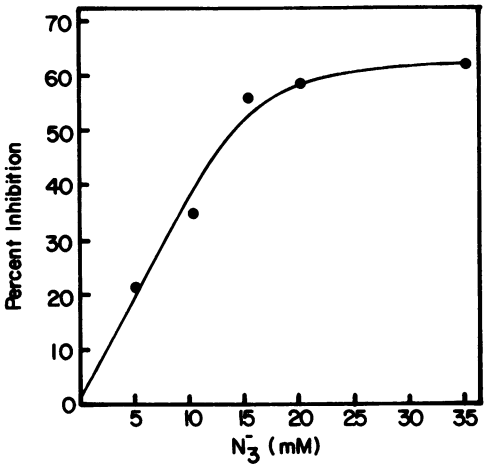

FIG. 3. Inhibition of pea leaf Mn SOD activity by azide. Different concentrations of azide were incorporated into the reaction mixture and the SOD activity was measured in 50 mM K-phosphate, 0.1 mM EDTA, pH 7.8, using the standard XOD-Cyt *c* assay (29).

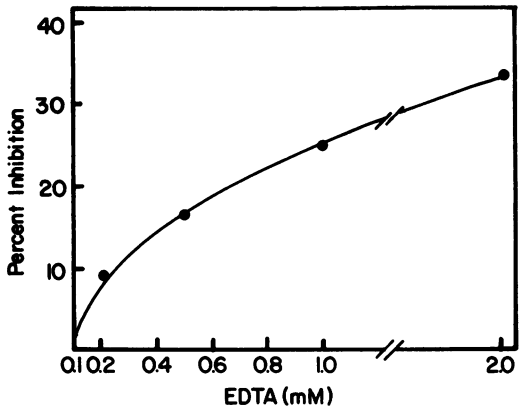

FIG. 4. Effect of EDTA on pea leaf Mn SOD activity. EDTA was added to the SOD reaction mixture and the enzymic activity was determined by the Cyt *c* method (29).

DISCUSSION

Mn SOD from *P. sativum* leaves as we have previously reported (41) is composed of four subunits of equal size, apparently bound by noncovalent forces, and its tetrameric structure is similar to the Mn SODs from chicken liver (30), bovine heart mitochondria (27), rat liver (34), human liver (30), yeast (33), the fungus *Pleurotus olearius* (21) and the bacteria *Thermus aquaticus* (37), *Thermus thermophilus* (38), and *Mycobacterium phlei* (7). The remaining Mn SODs so far fully characterized contain two subunits and occur in bacteria (15), including a photosynthetic organism (25), and in a red alga (31).

The amino acid composition of Mn SOD from leaves of *P.*

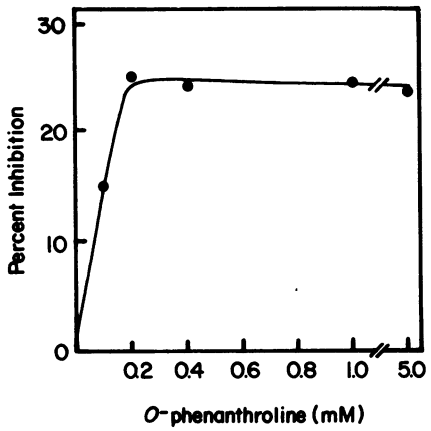

FIG. 5. Effect of *o*-phenanthroline on pea leaf Mn SOD activity. Conditions as for Fig. 4.

sativum show, like the majority of SODs belonging to this family, a predominance of aspartic acid and glutamic acid which is reflected in the slightly acidic value of the isoelectric point, 5.4, determined for this mangano enzyme (41). For purposes of comparison, in Table V the amino acid composition of Mn SODs isolated from organisms at different steps in the phylogenetic scale are presented, including *Escherichia coli* (42), *T. thermophilus* (38), *Rhodopseudomonas spheroides* (25), *Porphyridium cruentum* (31), yeast (33), rat liver (34), and human liver (30). As can be seen, the amino acid content of higher plant Mn SOD is comparable to the other enzymes in this class.

The method of Marchalonis and Weltman (26) for the determination of relatedness among proteins based upon analysis of least-squares differences in amino acid composition has permitted comparison of Mn SOD from a higher plant, with other Mn-containing SOD from different origins as well as with Fe- and Cu-Zn-containing SOD. This method can also provide indirect information on the degree of homology among primary sequence of proteins. The comparison of the Mn SOD from *P. sativum* leaves with other members of the same dismutase family showed that the highest degree of homology corresponded to Mn SODs from two eukaryotic nonphotosynthetic organisms which are phylogenetically distant from a higher plant: rat liver and yeast. The only Mn SODs so far characterized from photosynthetic organisms, the prokaryote *R. spheroides* (25) and the eukaryote *P. cruentum* (31), showed higher $S\Delta Q$ values. This indicates a higher degree of difference in the amino acid sequence between them and the Mn SOD from the eukaryotic photosynthetic organism *P. sativum*.

Comparison of the amino acid composition of the three types of SOD presented in Table II shows, in general, homology between Mn- and Fe-containing SODs, and a difference between these two families and Cu-Zn SOD. Particularly remarkable was the relatedness found between *P. sativum* Mn SOD and the Fe SOD from the higher plant *Brassica campestris* and the photosynthetic bacterium *Chromatium vinosum*, the former higher plant enzyme showing a homology degree similar to that determined for rat liver Mn SOD. The relatedness reported in this work between Mn and Fe SOD from a wide spectrum of organisms and the absence of homology between these enzymes and Cu-Zn SODs have also been described by Harris *et al.* (15) on the basis of the amino-terminal sequences determined in different SODs mainly from prokaryotic origin.

In general, Cu-Zn SODs display a similar enzymic activity at pH values between 7.8 and 10.2, whereas Fe and Mn SODs undergo a progressive decrease in activity at alkaline pH values (11). This pattern is also applicable to Mn SOD from pea leaves with an apparent pH optimum of about 8.6 but whose activity

Table V. *Amino Acid Composition of Mn-Containing Superoxide Dismutases from Different Origins*

Amino Acid	*E. coli* (42)	*T. thermophilus* (38)	*R. spheroides* (25)	*P. cruentum* (34)	Yeast (33)	*P. sativum*	Rat Liver (34)	Human Liver (30)
				residues/mole subunit				
Lysine	19	12	15	8	19	15	18	15
Histidine	6	8	8	8	7	8	8	9
Arginine	6	5	3	6	4	5	6	5
Aspartic acid	26	16	19	20	28	19	21	23
Threonine	10	7	8	13	11	12	10	5
Serine	10	3	11	9	8	14	12	6
Glutamic acid	18	20	22	11	28	20	24	22
Proline	8	12	7	8	10	10	12	10
Glycine	14	16	19	16	19	12	21	19
Alanine	24	18	18	18	21	20	17	17
Half-cystine	0	0	2	0	1	3	3	3
Valine	11	12	12	11	14	15	14	11
Methionine	2	3	4	2	1	2	3	3
Isoleucine	7	6	5	7	12	13	12	9
Leucine	19	20	15	12	20	20	21	16
Tyrosine	8	9	7	5	9	9	8	7
Phenylalanine	11	8	10	6	11	8	7	5
Tryptophan	5	6	4	3	6	8	6	5

Table VI. *Catalytic Activity of Different Superoxide Dismutases at 25°C and pH 7.8*

Enzyme	Rate Constant	Method Used	Reference
	$\times 10^{-9}$ ($M^{-1} \cdot s^{-1}$)		
Bovine Cu-Zn SOD	2.00	Pulse radiolysis	19
Bovine Cu-Zn SOD	1.90	Kinetic competition	11
E. coli Fe SOD	1.60	Kinetic competition	11
Photobacterium leiognathi Fe SOD	0.55	Pulse radiolysis	22
C. vinosum Fe SOD	2.40	Kinetic competition	17
S. platensis Fe SOD	1.60	Kinetic competition	25
B. stearothermophilus Mn SOD	0.60	Pulse radiolysis	28
R. spheroides Mn SOD	1.72	Kinetic competition	25
E. coli Mn SOD	1.80	Kinetic competition	11
Chicken liver Mn SOD	4.70	Kinetic competition	11
P. sativum Mn SOD	1.61	Kinetic competition	This work

diminishes at higher pH values. The few references available in literature on pH optimum values for SOD are close to that found for the pea mangano enzyme with the exception of Mn SOD from *P. olearius* (21) that shows a maximum catalytic efficiency at pH 10.

In Table VI, values are presented of second order rate constants for O_2^- disproportionation by different types of SOD determined at 25°C and pH 7.8 by using direct and indirect methods. The Mn SOD from pea leaves in its dismutating reaction of O_2^- radicals has a catalytic efficiency within the range described for other SODs studied. Its rate constant (1.61×10^9 M^{-1} s^{-1}) is identical with that reported for Fe SOD from *E. coli* (11) and the blue-green algae *Spirulina platensis* (25) and of a similar order of magnitude to the rate constants determined for Mn SOD from *E. coli* (11) and from the photosynthetic non-sulfur bacterium *R. spheroides* (25).

The pea Mn SOD contains 1 atom of Mn/tetramer of 94,500 mol wt (41), an unusual stoichiometry for a Mn SOD. The enzymes of this type so far characterized contain either 1 atom Mn/dimer, 2 atoms/tetramer, or 4 atoms/tetramer. In the first category are classified Mn SODs from a number of bacteria (15), including a photosynthetic organism (25), and a red alga (31). Mn SODs containing 2 atoms Mn/tetramer are those of bacterial and fungal origin (21, 37, 38), chicken liver (33), and bovine heart mitochondria (27), whereas enzymes with 4 atoms Mn/tetramer are restricted to yeast (33) and mammals (30, 34).

It should be noted that Mn SOD from pea leaves, Fe SOD from *E. coli* (43) and from *S. platensis* (25), and Mn SOD from *E. coli* (43) and from *R. spheroides* (25) all contain 1 metal atom/enzyme molecule. Those dismutases of higher catalytic efficiency such as chicken liver Mn SOD (11), *C. vinosum* Fe SOD (17), and bovine erythrocyte Cu-Zn SOD (11) have metal contents per molecule of 2 atoms Mn, 2 atoms Fe, and 2 atoms of Cu and Zn, respectively. This suggests the existence of a relationship between the catalytic activity and the number of metal atoms present in their molecules, an idea that seems consistent with the mechanism of action of these enzymes, implying the alternative reduction and reoxidation of the enzyme metal (Cu^{2+}, Mn^{3+}, or Fe^{3+}) during its successive encounters with O_2^- radicals (13).

Treatment of pea Mn SOD with PHMB, a typical inhibitor of -SH groups, brings about a nearly complete inhibition of enzymic activity at 1 mM final concentration. On the contrary, Fe SOD from *Plectonema boryanum* is insensitive to PHMB at a similar concentration (3) whereas Cu-Zn SOD from spinach leaves, under these conditions, is inhibited by 35% (2). This is most likely a reflection of the greater number of half-cystine residues present in the mangano enzyme.

Azide also inhibits the activity of the pea mangano enzyme, leading to inhibition rates of more than 60% at a concentration of 35 mM. A concentration of 10 mM led to about a 38% inhibition of pea Mn SOD activity at pH 7.8, a result nearly identical with that described for Mn SOD from the red algae *P. cruentum* (31) and the bacterium *Bacillus stearothermophilus* (28).

The metal chelators assayed with pea Mn SOD (*o*-phenanthroline, EDTA, and DIECA) all slightly inhibited the enzymic activity in decreasing order of intensity. Phenanthroline at 1 mM concentration brings about a 25% inhibition whereas Cu-Zn SOD from spinach leaves, under similar conditions, is inhibited by 38% (2). The Cu(II) chelator DIECA, a powerful inhibitor of Cu-Zn SOD, inactivates pea Mn SOD approximately 24% at a 10 mM concentration, whereas under similar assay conditions, the activity of spinach leaf Cu-Zn SOD is inhibited in 68% (2), and in other Cu-Zn SODs from animal origin, the activity can be even completely repressed both *in vitro* and *in vivo* by the action of this inhibitor (16).

In the light of the different metal-protein stoichiometries of the Mn SODs presently known, it may be of significance to note that those enzymes characterized from photosynthetic organisms, the bacterium *R. spheroides* (25) and the red alga *P. cruentum* (31), have 1 atom Mn/molecule. However, this property appears to be one of the few similarities between pea leaf Mn SOD and photosynthetic Mn SODs. In fact, our least-squares difference analysis of amino acid composition of different SODs shows that Mn SOD from rat liver and yeast have the highest degree of homology with pea leaf Mn SOD. The subcellular location of those enzymes is known to be the mitochondrial matrix of the respective organisms (33, 34). In experiments carried out in our laboratory to elucidate the cell location of SOD isozymes in pea leaves, we repeatedly found the Mn SOD isozyme present in the mitochondrial fraction, whereas in chloroplasts only a Cu-Zn SOD was present (unpublished work). These results coincide with those previously described by Foster and Edwards (12).

The Mn SOD from the higher plant *P. sativum* adds to a wide spectrum of Mn SODs characterized from diverse organisms including bacteria, yeast, algae, fungi, and animals, and permits the establishment of phylogenetic comparisons between this higher plant enzyme and different SODs from photosynthetic and nonphotosynthetic organisms. The knowledge of the molecular properties of this Mn SOD will allow initiating studies aimed at the elucidation of its precise physiological function in the plant cell at organelle level, a subject on which we are concentrating our attention.

Acknowledgments—The authors are indebted to Dr. M. Gómez-Ortega for the growth of pea plants under greenhouse conditions and to Miss M. Garrido-Ortíz and Mr. A. Melgar-Porcel for skillful technical assistance. Dr. Marvin L. Salin's (Mississippi State University) valuable help in reviewing the manuscript is acknowledged.

LITERATURE CITED

1. ASADA K, M URANO, M TAKAHASHI 1973 Subcellular location of superoxide dismutase in spinach leaves and preparation and properties of crystalline spinach superoxide dismutase. Eur J Biochem 36: 257–266
2. ASADA K, M TAKAHASHI, M NAGATE 1974 Assay and inhibitors of spinach superoxide dismutase. Agr Biol Chem 38: 471–473
3. ASADA K, K YOSHIKAWA, M TAKAHASHI, Y MAEDA, K ENMANJI 1975 Superoxide dismutases from a blue-green alga, *Plectonema boryanum* J Biol Chem 250: 2801–2807
4. BAUM JA, JG SCANDALIOS 1981 Isolation and characterization of the cytosolic and mitochondrial superoxide dismutases of maize. Arch Biochem Biophys 206: 249–264
5. BEAUCHAMP CO, I FRIDOVICH 1971 Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44: 276–287
6. BEAUCHAMP CO, I FRIDOVICH 1973 Isozymes of superoxide dismutase from wheat germ. Biochim Biophys Acta 317: 50–64
7. CHIKATA Y, E KUSUNOSE, K ICHIHARA, M KUSUNOSE 1975 Purification of superoxide dismutases from *Mycobacterium phlei.* Osaka City Med J 21: 127–136
8. DEL RIO LA, F SEVILLA, M GOMEZ, J YAÑEZ, J LOPEZ-GORGE 1978 Superoxide dismutase: an enzyme system for the study of micronutrient interactions in plants. Planta 140: 221–225
9. DEL RIO LA, M GOMEZ, J YAÑEZ, A LEAL, J LOPEZ-GORGE 1978 Iron deficiency in pea plants. Effect on catalase, peroxidase, chlorophyll and proteins of leaves. Plant Soil 49: 343–353
10. EDELHOCH H 1967 Spectroscopic determination of tryptophan and tyrosine in proteins. Biochemistry 6: 1948–1954
11. FORMAN HJ, I FRIDOVICH 1973 Superoxide dismutase: a comparison of rate constants. Arch Biochem Biophys 158: 396–400
12. FOSTER JG, GE EDWARDS 1980 Localization of superoxide dismutase in leaves of C_3 and C_4 plants. Plant Cell Physiol 21: 895–906
13. FRIDOVICH I 1978 The biology of oxygen radicals. Science (Wash DC) 201: 875–880
14. GARCIA JE, M GOMEZ, J YAÑEZ, J LOPEZ-GORGE, LA DEL RIO 1981 Isozyme pattern of the metalloenzyme system superoxide dismutase during growth of peas (*Pisum sativum* L.) under different iron nutrient concentrations. Z Pflanzenphysiol 105: 21–29
15. HARRIS JI, AD AUFFRET, FD NORTHROP, JE WALKER 1980 Structural comparisons of superoxide dismutases. Eur J Biochem 106: 297–303
16. HEIKKILA RE, G COHEN 1977 The inactivation of copper-zinc superoxide dismutase by diethyldithiocarbamate. *In* AM Michelson, JM McCord, I Fridovich, eds, Superoxide and Superoxide Dismutases. Academic Press, New York, pp 367–373
17. KANEMATSU S, K ASADA 1978 Superoxide dismutase from an anaerobic photosynthetic bacterium, *Chromatium vinosum.* Arch Biochem Biophys 185: 473–482
18. KANEMATSU S, K ASADA 1979 Ferric and manganic superoxide dismutases in *Euglena gracilis.* Arch Biochem Biophys 195: 535–545
19. KLUG D, J RABANI, I FRIDOVICH 1972 A direct demonstration of the catalytic action of superoxide dismutase through the use of pulse radiolysis. J Biol Chem 247: 4839–4842
20. KONO Y, M TAKAHASHI, K ASADA 1979 Superoxide dismutases from kidney bean leaves. Plant Cell Physiol 20: 1229–1235
21. LAVELLE F, AM MICHELSON 1975 Purification et étude des deux superoxyde dismutases du champignon *Pleurotus olearius.* Biochimie 57: 375–381
22. LAVELLE F, ME MCADAM, EM FIELDEN, PB ROBERTS, K PUGET, AM MICHELSON 1977 A pulse-radiolysis study on the catalytic mechanism of the iron-containing superoxide dismutase from *Photobacterium leiognathi.* Biochem J 161: 3–11
23. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin-phenol reagent. J Biol Chem 193: 265–275
24. LUMSDEN J, DO HALL 1974 Soluble and membrane-bound superoxide dismutases in a blue-green alga (Spirulina) and spinach. Biochem Biophys Res Commun 58: 35–41
25. LUMSDEN J, R CAMMACK, DO HALL 1976 Purification and physicochemical properties of superoxide dismutase from two photosynthetic microorganisms. Biochim Biophys Acta 438: 380–392
26. MARCHALONIS JJ, JK WELTMAN 1971 Relatedness among proteins: a new method of estimation and its application to immunoglobulins. Comp Biochem Physiol 38B: 609–625
27. MARKLUND S 1978 Purification and characterization of a manganese containing superoxide dismutase from bovine heart mitochondria. Int J Biochem 9: 299–306
28. MCADAM ME, F LAVELLE, RA FOX, E MARTIN-FIELDEN 1977 A pulse-radiolysis study of the manganese-containing superoxide dismutase from *Bacillus stearothermophilus.* Further studies on the properties of the enzyme. Biochem J 165: 81–87
29. MCCORD JM, I FRIDOVICH 1969 Superoxide dismutase: an enzymic function for erythrocuprein. J Biol Chem 244: 6049–6055
30. MCCORD JM, JA BOYLE, ED DAY, LJ RIZZOLO, ML SALIN 1977 A manganese-containing superoxide dismutase from human liver. *In* AM Michelson, JM McCord, I Fridovich, eds, Superoxide and Superoxide Dismutases. Academic Press, New York, pp 129–138
31. MISRA HP, I FRIDOVICH 1977 Purification and properties of superoxide dismutase from a red alga, *Porphyridium cruentum.* J Biol Chem 252: 6421–6423
32. MOORE S 1963 The determination of cystine as cysteic acid. J Biol Chem 238: 235–237
33. RAVINDRANATH SD, I FRIDOVICH 1975 Isolation and characterization of a manganese-containing superoxide dismutase from yeast. J Biol Chem 250: 6107–6112
34. SALIN ML, ED DAY, JD CRAPO 1978 Isolation and characterization of a manganese-containing superoxide dismutase from rat liver. Archiv Biochem Biophys 187: 223–228
35. SALIN ML, SM BRIDGES 1980 Isolation and characterization of an iron-containing superoxide dismutase from a eucaryote, *Brassica campestris.* Arch Biochem Biophys 201: 369–374
36. SALIN ML, SM BRIDGES 1982 Isolation and characterization of an iron-containing superoxide dismutase from water lily, *Nuphar luteum.* Plant Physiol 69: 161–165
37. SATO S, JI HARRIS 1977 Superoxide dismutase from *Thermus aquaticus.* Isolation and characterization of manganese and apo enzymes. Eur J Biochem 73: 373–381
38. SATO S, K NAKAZAWA 1978 Purification and properties of superoxide dismutase from *Thermus thermophilus* HB8. J Biochem (Tokyo) 83: 1165–1171
39. SAWADA Y, T OHYAMA, I YAMAZAKI 1972 Preparation and physicochemical properties of green pea superoxide dismutase. Biochim Biophys Acta 268: 305–312
40. SEVILLA F, J LOPEZ-GORGE, M GOMEZ, LA DEL RIO 1980 Manganese superoxide dismutase from a higher plant. Purification of a new Mn-containing enzyme. Planta 150: 153–157
41. SEVILLA F, J LOPEZ-GORGE, LA DEL RIO 1980 Preliminary characterization of a Mn-containing superoxide dismutase from a higher plant (*Pisum sativum* L.). *In* JV Bannister, HAO Hill, eds, Chemical and Biochemical Aspects of Superoxide and Superoxide Dismutase. Elsevier/North-Holland, New York, pp 185–195
42. STEINMAN HM 1978 The amino acid sequence of mangano superoxide dismutase from *Escherichia coli* B. J Biol Chem 253: 8708–8720
43. YOST FJ, I FRIDOVICH 1973 An iron-containing superoxide dismutase from *Escherichia coli.* J Biol Chem 248: 4905–4908