Free Rad. Res. Comms., Vols. 12-13, pp. 319-328
Reprints available directly from the publisher
Photocopying permitted by license only

PURIFICATION OF AN IRON-CONTAINING SUPEROXIDE DISMUTASE FROM A CITRUS PLANT, *CITRUS LIMONUM R*

MARÍA S. ALMANSA, JOSÉ M. PALMA*, JUAN YÁÑEZ*, LUIS A. DEL RÍO* and FRANCISCA SEVILLA

Unidad de Alimentación y Desarrollo de la Planta, Centro de Edafología y Biología Aplicada del Segura, CSIC, Apdo 195, E-30080 Murcia, Spain;
**Unidad de Bioquímica Vegetal, Estación Experimental del Zaidín, CSIC, Apdo 419, E-18080 Granada, Spain*

A cyanide-insensitive superoxide dismutase was purified to apparent homogeneity from lemon leaves (*Citrus limonum R*). The enzyme was isolated from leaf extracts by ammonium sulfate salting-out, and ion-exchange, gel filtration and hydroxylapatite column chromatography. The purified Fe-SOD had a specific activity of about 1,500 U/mg and represents approximately 1.6% of the total soluble protein in lemon leaf extracts. A molecular weight of 47,500 was determined for the enzyme. Analytical gel electrofocusing of the purified preparation revealed the presence of two isozymes with pI values of 5.13 and 4.98. Metal analysis showed the presence of 1 g-atom of iron and 0.5 g-atom of manganese per mol of enzyme. The visible and UV absorption spectra of the Citrus enzyme were similar to those reported for other iron-containing SODs from different origins. The significance of the presence of Fe-SOD in higher plants is briefly discussed.

KEY WORDS: Citrus, evolution, iron, lemon, purification, superoxide dismutase.

INTRODUCTION

Superoxide dismutases (SOD; EC 1.15.1.1) are a family of metalloenzymes that catalyze the disproportionation of potentially harmful superoxide free radicals (O_2^-) to H_2O_2 and O_2.[1] Iron-containing superoxide dismutases are mainly present in procaryotic organisms and some eucaryotic algae, but have also been found in the plant families, *Gingkoaceae, Nymphaceae,* and *Cruciferae*,[2] and in leaves of beans and tomatoes.[3] In previous papers, we provided evidence of the presence of two Fe-SODs in *Citrus limonum* and proposed the use of the metalloenzyme system SOD as a biological marker in nutritional studies in lemon plants.[4,5] Recently, the occurrence of iron-containing superoxide dismutases in other species of the plant family *Rutaceae*, as well as in nodules of *Vigna unguiculata*, and in species of the plant families *Rubiaceae, Caryophyllaceae* and *Solanaceae* has also been demonstrated.[6-10] Therefore, it seems that Fe-SOD has a wider distribution in the plant kingdom than was previously thought.

This work describes the purification and some properties of a *Citrus* Fe-SOD. This is the first report of the preliminary characterization of a Fe-SOD belonging to the plant family *Rutaceae* which is phylogenetically close to some plant families where this type of SOD has been recently found.

Correspondence: Prof. Luis A. del Río, Unidad de Bioquímica Vegetal, Estación Experimental del Zaidín (CSIC), Apdo 419, E-18080 Granada, Spain.

MATERIALS AND METHODS

Lemon leaves (*Citrus limonum* R.) were obtained from trees grown by strip irrigation under field conditions at the CSIC experimental farm. SOD activity was determined by the method of McCord and Fridovich[11] except for column eluates, where the assay of the photochemical reduction of NBT[12] was used. Polyacrylamide gel electrophoresis was carried out on 10% gels. Isozymes of SOD were visualized on gels using the photochemical method.[13] The three types of SOD were identified by performing the activity stains in gels previously incubated at 25° C for 45 min in 50 mM K-phosphate, pH 7.8, 0.1 mM EDTA, containing either 5 mM H_2O_2 or 2 mM CN^-. Electrofocusing was conducted in 7.5% acrylamide gels containing ampholytes of a 1:0.73 mixture of Pharmalytes (Pharmacia) pH 4.2–4.9 and pH 4.5–5.4, respectively. Protein content of crude extracts and purified fractions was determined according to Potty[14] and Murphy and Kies[15] respectively. All spectrophotometric measurements and the scanning of polyacrylamide gels were performed on a Shimadzu UV-260 recording spectrophotometer. The molecular weight was determined by gel filtration on Sephadex G-100 using the following Mr standards: bovine serum albumin (66,000), ovalbumin (45,000), trypsinogen (24,000), and cytochrome *c* (12,400). Metal content was determined by atomic absorption spectrophotometry using a Perkin-Elmer 5000 apparatus equipped with a heated graphite atomizer, model HGH-400.

RESULTS

Purification of Fe-SOD

Unless otherwise stated all the purification procedures were performed at 0–4° C. Lemon leaves (1.1 Kg) previously washed were cut into short segments and blended in 50 mM K-phosphate, pH 7.8, containing 0.1 mM EDTA, 5 mM cysteine, 1 mM phenylmethylsulfonyl fluoride, 1% PVP and 0.2% Triton X-100, using a polytron tissue homogenizer. Homogenates were filtered through four layers of nylon cloth and centrifuged at 8,000 g for 15 min. The supernatant was brought to 30% saturation with ammonium sulfate. After stirring for 2 h the suspension was centrifuged at 10,500 g for 20 min, and ammonium sulfate was added to the supernatant to 80% saturation. The pellet collected by centrifugation was dissolved in about 215 ml of 10 mM K-phosphate, pH 7.8, 0.3 mM DTT and dialyzed for 50 h against the same buffer. The dialyzed sample was clarified by centrifugation and mixed for 120 min with 240 ml of DEAE-Trisacryl (Pharmacia) equilibrated in 10 mM K-phosphate, pH 7.8, 0.3 mM DTT, and then centrifuged at 12,000 g for 30 min. The supernatant contained about 15% of the total cyanide-insensitive and H_2O_2-sensitive Fe-SOD activity. The adsorbed cyanide-insensitive activity (Fe-SOD plus Mn-SOD) was eluted with 0.8 M NaCl in 10 mM K-phosphate, pH 7.8, 0.3 mM DTT, and then was dialyzed against the same buffer. The sample was concentrated to 80 ml and loaded into a column of DEAE-Trisacryl (2.5 × 48 cm) equilibrated in 10 mM K-phosphate, pH 7.8, 0.3 mM DTT, and was washed with the same buffer (2 l) until absorption at 280 nM decreased below 0.4. The adsorbed sample was eluted with a 1.5 l linear gradient of NaCl (0–0.4 M). This allowed the separation of three SOD activity peaks: Fraction 1, containing isozyme Fe-SOD I and Cu,Zn-SODs; Fraction 2, containing Fe-SOD II; and Fraction 3, containing the bulk of Mn-SOD activity and part of

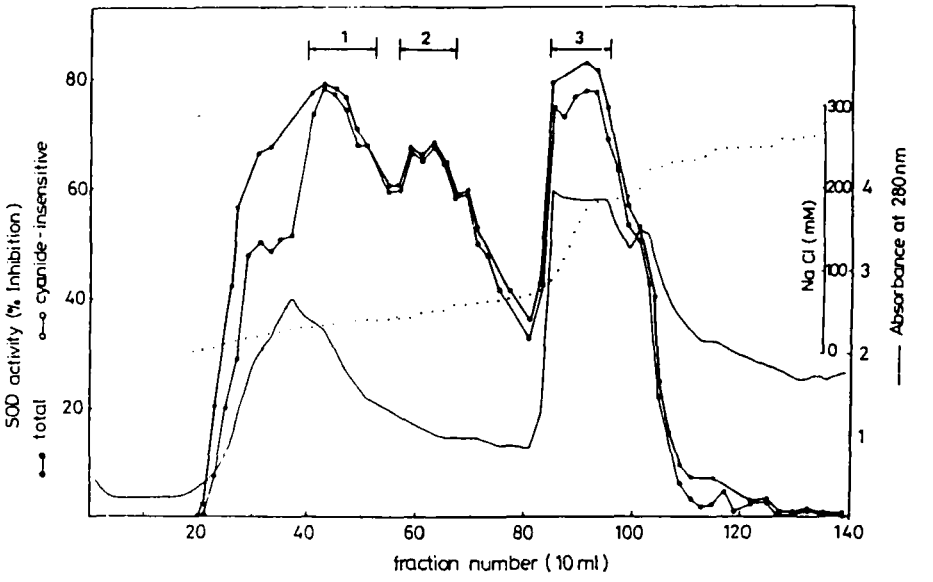

FIGURE 1 Separation of superoxide dismutases of *C. limonum* by ion-exchange chromatography on DEAE-Trisacryl. The dialyzed enzyme solution from the DEAE-Trisacryl batch step was applied to a column of DEAE-Trisacryl equilibrated in 10 mM K-phosphate, pH 7.8, 0.3 mM DTT, and eluted as indicated in the text. The horizontal bars (1,2, and 3) represent the SOD activity peaks which were pooled separately. The gradient of NaCl used is depicted as a dotted line (. . . . .). SOD activity is expressed as the percentage of inhibition of the photochemical reduction of NBT (12).

Fe-SOD II (Figure 1). Each peak was pooled separately, dialyzed against 50 mM K-phosphate, pH 7.8, 0.3 mM DTT and concentrated to 10 ml. For the purification, fraction I was chosen because Fe-SOD I had the highest specific activity. This sample was applied to a column of Sephadex G-100 (2.5 × 100 cm) equilibrated in 50 mM K-phosphate, pH 7.8, 0.3 mM DTT and was eluted with the same buffer. Active fractions were pooled and dialyzed against 10 mM K-phosphate, pH 7.8, 0.3 mM DTT and then concentrated by ultrafiltration on an Amicon PM-10 membrane. The enzyme solution (6 ml) was applied to a column of hydroxylapatite (1.6 × 20 cm) equilibrated in 10 mM K-phosphate, pH 7.8, 0.3 mM DTT. The adsorbed Fe-SOD was eluted with a 300 ml linear gradient of phosphate (0.01–0.08 M) at a concentration range of 29–48 mM. Finally, the enzyme preparation was concentrated by ultrafiltration and subjected to gel filtration chromatography using a column of Sephadex G-100 (1.5 × 100 cm) equilibrated in 50 mM K-phosphate, pH 7.8, containing 0.3 mM DTT and 0.1 mM EDTA. Active fractions were concentrated by ultrafiltration and stored either precipitated with $(NH_4)_2SO_4$ at 2° C or frozen at −20° C.

Table I summarizes the results of the purification procedure. The DEAE-Trisacryl batch step was essential for the removal of a considerable portion of cyanide-sensitive superoxide dismutase activity. DEAE-Trisacryl column step (Figure 1) allowed the separation of isozyme Fe-SOD I and a portion of Fe-SOD II from Mn-SODs. However two isozymes of Cu,Zn-SOD remained bound to the column along with the Fe-SOD I and, consequently, some chromatographic fractions of this iron-containing isozyme had to be discarded due to the contamination by the cuprozinc enzymes.

TABLE I
Purification of an iron-containing SOD from leaves of *Citrus limonum R.*

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude supernatant	851,333	36,556	23	100	1
30–80% $(NH_4)_2SO_4$	445,021	7,752	57	52	2.5
DEAE-Trisacryl batch step	168,892	1,807	93	20	4.1
Gradient DEAE-triscryl					
I	11,006	57	193	1.3	8.5
II	43,505	574	76	5.1	3.3
III	95,489	899	106	11.2	4.7
Sephadex G-100					
I	7,330	16	458	0.9	20
Hydroxylapatite					
I	4,607	3.6	1,294	0.5	57
Sephadex G-100					
I	3,643	2.5	1,457	0.4	64

[a]For the purification a total amount of 1,100 g of fresh lemon leaves were used.
[b]SOD activity was determined in the presence of 1 mM KCN.

Moreover, Sephadex G-100 chromatography failed to remove the small amount of a Cu,Zn-SOD which was copurified until the hydroxylapatite column step, where this contaminating cyanide-sensitive activity was eliminated.

The purified Fe-SOD I had a specific activity of about 1,500 U/mg. This value is lower than that reported for some procariotic Fe-SODs and for the enzyme from *Lycopersicon esculentum* ,[16-18] but is similar to that described for other eukaryotic Fe-SODs.[19,20] The enzyme was purified 64-fold and a yield of about 0.4% was obtained. It can be estimated that Fe-SOD I represents approximately 1.6% of the total soluble proteins in lemon leaf extracts. By electrophoresis in 10% gels a single protein band was detected which coincided with the band of activity (Figure 2). In contrast, electrofocusing revealed charge heterogeneity, showing the presence of two isozymes with pI values of 4.98 and 5.13. Several bands of isozymic activity have also been found in purified Fe-SODs from *Nuphar* and *Ginkgo*.[20,21]

Molecular weight

The molecular weight determined for the purified enzyme by gel-exclusion chromatography yielded a value of 47,500, as it is shown in Figure 3.

Absorption spectra

Figure 4 shows the visible and ultraviolet absorption spectra of the purified enzyme. It showed an absorption maximum at 278 nm with two slight shoulders at 260 nm and 290 nm. A broad, weak absorption in the range of 300–450 nm with two shoulders at 340 nm and 388 nm, were also observed. At 278 nm a molar extinction coefficient (ε_{278}) of $5.6 \times 10^4 M^{-1} cm^{-1}$ was calculated, a value similar to that reported for the Fe-SODs from *E. Coli*,[22] *Nuphar*,[20] *Gingko*[21] and *Brassica*.[19]

Metal analysis

The purified enzyme was exhaustively dialyzed first against 5 mM K-phosphate, pH

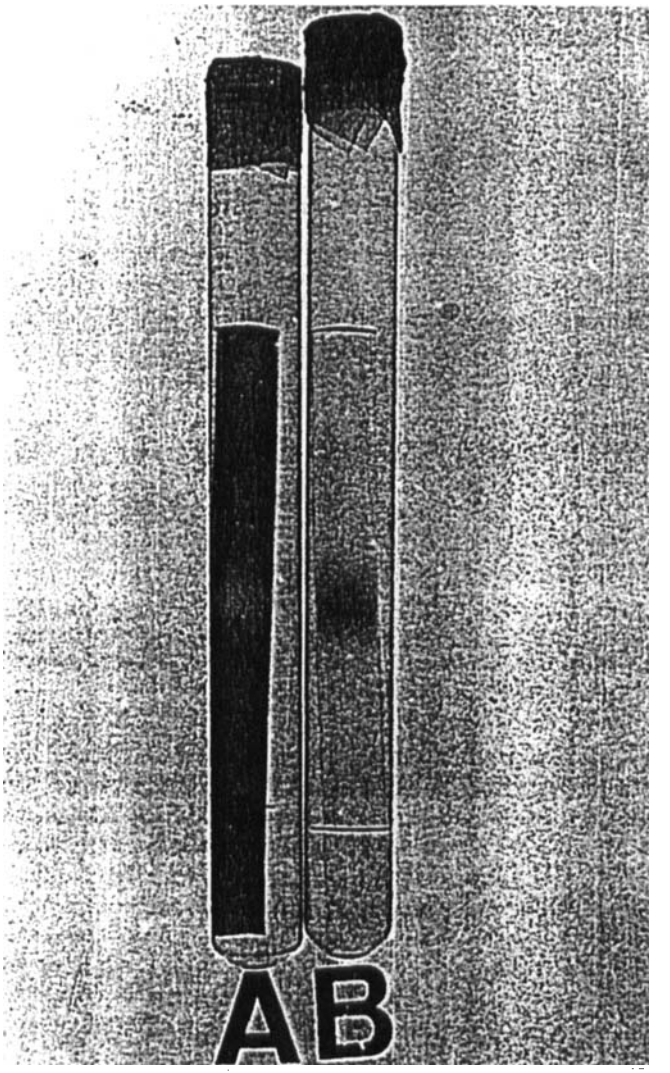

FIGURE 2 Polyacrylamide gel electrophoresis of Fe-SOD I purified from *C. limonum*. A, 30 μg protein stained for SOD activity with NBT. B, 20 μg protein stained with Coomassie blue.

7.8, containing 0.1 mM EDTA, and then against this buffer lacking EDTA. The enzyme was assayed for manganese, iron and copper by atomic absorption spectroscopy. Results obtained from two different purification batches showed the presence of 1.0 g-atom of iron and 0.5 g-atom of manganese per mol of enzyme (Table II), while copper content was lower than 0.3 g-atoms per mol.

DISCUSSION

The preliminary characterization of purified Fe-SOD I from leaves of *C. limonum* revealed similarities in molecular properties to previously purified Fe-SODs from

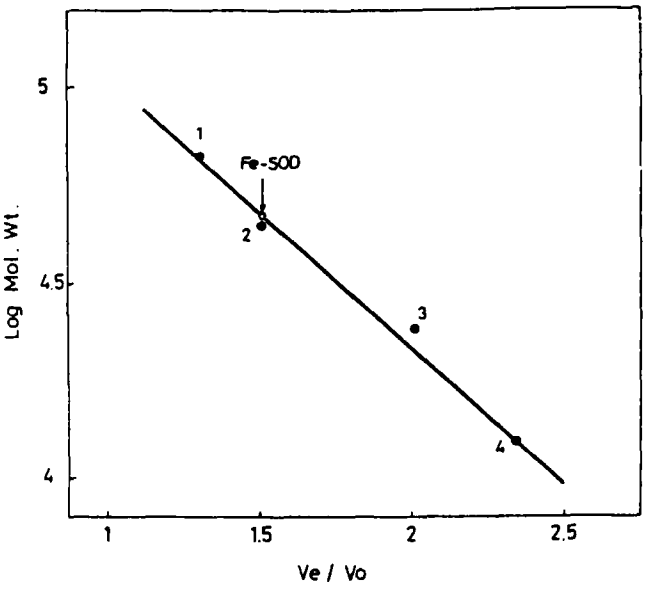

FIGURE 3 Determination of the molecular weight of Fe-SOD I from *C. limonum*. The enzyme was passed through a Sephadex G-100 column (2.5 × 95 cm) equilibrated in 50 mM K-phosphate. pH 7.8. The standard proteins used were: 1, bovine serum albumin (66,000); 2, ovalbumin (45,000); 3, trypsinogen (24,000); 4, cytochrome c (12,400). V_0, void volume; Ve, elution volume.

both procaryotic and eucaryotic sources. The molecular weight of the enzyme is in the range 40,000–47,000 reported for other Fe-SODs,[16–24] and its metal content is similar to that described for other iron-containing superoxide dismutases, which fluctuates between 1 and 2 g-atoms Fe per mol.[20,21] The visible and UV absorption spectra of *Citrus limonum* Fe-SOD are similar to those reported for other Fe-SODs.[17,18] The low yields obtained for lemon iron-superoxide dismutase are comparable to those described for the Fe-SODs from *Brassica*[19] and *Gingko*.[21] In all these cases, apart from a specifically high unstability of these Fe-enzymes, an explanation for the poor recoveries obtained could be the difficulties encountered in separating Cu,Zn- and Mn-SODs from Fe-SOD. We found that due to the similarities, either in charge or molecular weight, between Cu,Zn SODs and Fe-SODs present in lemon leaf extracts, part of Fe-SOD I was contaminated by copperzinc-SODs which were copurified until the hydroxylapatite column step.

In higher plants, Cu,Zn-SODs occur mainly within chloroplasts[25,26] but they also have been located in the cytosol, mitochondria, and in glyoxysomes[27–29] Fe-SODs appear to be located in chloroplasts[18,30] and Mn-SODs are mainly present in mitochondria.[31] More recently, the occurrence of Mn-SOD in pea leaf peroxisomes and of Mn-SOD plus Fe-SOD in peroxisomes from carnation petals, has also been reported.[32,33] The study of the intracellular distribution of Fe-SODs in lemon leaves is now under way in our laboratory, in order to see whether the different Fe-containing isozymes are located exclusively in chloroplasts or are also distributed in other cell organelles. In chloroplasts, mitochondria, and peroxisomes, the production of O_2^- has been clearly demonstrated and SOD activity appears to play an important role in maintaining the correct oxidative equilibrium of these cellular organelles.[30,31,34,35]

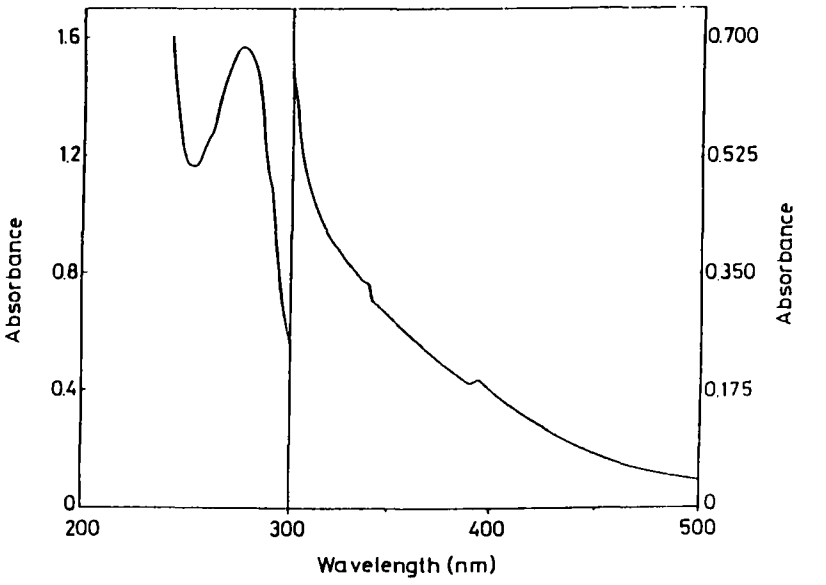

FIGURE 4 Absorption spectrum of Fe-superoxide dismutase from *C. limonum*. The enzyme concentration was 0.235 mg/ml.

Nutritional studies of SOD in higher plants have demonstrated that stress situations in certain micronutrients, either deficiency or toxicity, can determine the expression of SODs. Results obtained suggest that the biosynthesis of SOD isozymes is interdependent and is probably coordinately controlled, and imply that the production of O_2^--derived toxic species by an increase in the intracellular levels of metals can determine the balance between these SODs.[4,5,36,37] Recently, the detection of a Fe-SOD isozyme in Cu-depleted leaves of *Pisum sativum* and the parallel induction and depression of Mn-SOD and Cu,Zn-SODs, respectively, has been described.[38] These results show that this Fe-SOD, which is not found in pea plants under normal nutrient conditions, could be induced as a result of Cu-restriction. In plants, copper deficiency is associated, among other effects, with diminished amounts of plastocyanin.[38] This copper-protein plays an important function as the primary electron donor of PS-I, the main source of O_2^- radicals in chloroplasts.[39]

The induction of Fe-SODs by Fe(II) in Fe-deficient lemon leaves was demonstrated and the possible involvement of O_2^- in the induction of Fe-SOD, as a protection of chloroplasts against the restored production of these radicals by PS-I, was also

TABLE II
Metal composition of Fe-SOD from *C. linonum*

Metals	g-atoms/mol enzyme[a]
Fe	1.04
Mn	0.50

[a]Values are means of two determinations with samples from different preparations of the purified enzyme.

suggested.[40] On the other hand, in leaves from tomato plants subjected to cold treatment, the temporary loss of chloroplast Cu,Zn-SOD activity and the unalteration of the chloroplastic Fe-SOD activity seems to indicate that, under this stress situation, Fe-SOD might take over the function of Cu,Zn-SOD.[18] Likewise, the induction of SOD isozymes in response to increased rates of intra cellular O_2^- production by other environmental factors, has also been described.[31,41]

The identification of Fe-SODs in plant families of different evolutionary branches but some of which are phylogenetically close, is indicative that these plant species, unlike many other organisms, have the genetic potential for expressing the three types of SOD. Among the evolutive hypotheses formulated to explain the presence of Fe-SODs in plants, the possibility of gene transfer from bacteria or algae to plant families,[2] seems to be the least plausible. It appears more likely that the presence of Fe-SOD in higher plants could be due to the expression of silent genes coding for Fe-SODs, as a result of environmental pressures, a hypothesis that has been previously formulated to explain the distribution of iron-containing SODs in different higher plants.[2]

However, the question of why these plants have expressed an iron enzyme instead of enhancing the production of an already present chloroplastic Cu,Zn-SOD, is something that remains to be explained. Results from nutritional and environmental stress studies suggest that the expression of Fe-SOD in plants could be regulated by distinct control factors related to the plant ability to develop adaptive protection responses against different types of stress conditions. Thus, as pointed out by Kwiatowsky *et al.* (1985), organisms containing two different SODs in chloroplasts could have an evolutionary advantage.

Acknowledgments

The authors are grateful to Mrs. D. Lapaz for excellent technical assistance. This work was supported by grant PB87-0404-02 from the DGICYT (Spain).

References

1. I. Fridovich (1986) Biological effects of the superoxide radical. *Arch. Biochem. Biophys.*, **247**, 1–11.
2. S.M. Bridges and M.L. Salin (1981) Distribution of iron-containing superoxide dismutase in vascular plants. *Plant Physiol.*, **68**, 275–278.
3. J. Kwiatowski and Z. Kaniuga (1984) Evidence for iron-containing superoxide dismutase in leaves of *Lycopersicon esculentum* and *Phaseolus Vulgaris*. *Acta Physiol. Plant.*, *6*, 197–202.
4. F. Sevilla, L.A. del Río and E. Hellín (1984a) Superoxide dismutases from a Citrus plant: Presence of two iron-containing isoenzymes in leaves of lemon trees (*Citrus limonum* R.). *J. Plant Physiol.*, **116**, 381–387.
5. F. Sevilla, E. Hellín, C.F. Alcaraz and S. Llorente (1984b) Superoxide dismutase activities in the diagnosing of trace element deficiencies in Citrus plants. In *Proc. VIth Int. Colloquium Optimization Plant Nutrition*, Vol. II (ed. P. Martin-Prevel), Aionp-Gerdat, Montpellier, pp. 625–633.
6. M.S. Almansa, L.A. del Río, C.F. Alcaraz and F. Sevilla (1989) Isoenzyme pattern of superoxide dismutase in different varieties of Citrus plants. *Physiol. Plant.*, **76**, 563–568.
7. M. Becana, F.J. Paris, L.M. Sandalio and L.A. del Río (1989) Isoenzymes of superoxide dismutase in nodules of *Phaseolus vulgaris* L., *Pisum sativum* L., and *Vigna unguiculata* (L) Walp. *Plant Physiol.*, **90**, 1286–1292.
8. M.C. Daza, L.M. Sandalio, L.A. del Río and M. Quijano-Rico (1989) Superoxide dismutases from Rubiaceae plants: Presence of an iron-containing isoenzyme in leaves of *Coffea arabica* L. In preparation.
9. M.J. Droillard, D. Bureaux, A. Paulin and J. Daussant (1989) Identification of different classes of superoxide dismutase in carnation petals. *Electrophoresis*, **10**, 46–48.

10. W. Van Camp, C. Bowler, M. Van Montagu and D. Inzé (1989) A simple and very efficient method to isolate cDNAs encoding superoxide dismutases: Isolation of an Fe-SOD from plants. *Fifth Conference on Superoxide and Superoxide Dismutase* (Jerusalem). Abstracts, p. 126.
11. J.M. McCord and I. Fridovich (1969) Superoxide dismutase. An enzymatic function for erythrocuprein (hemocuprein). *J. Biol. Chem.*, **244**, 6049–55.
12. C.N. Giannopolitis and S.K. Ries (1977) Superoxide dismutases. I. Occurrence in higher plants. *Plant Physiol.*, **59**, 309–314.
13. C.O. Beauchamp and I. Fridovich (1971) Superoxide dismutase improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.*, **44**, 276–287.
14. V.H. Potty (1969) Determination of protein in the presence of phenols and pectins. *Anal. Biochem.*, **29**, 535–539.
15. J.B. Murphy and M.W. Kies (1960) Note on spectrophotometric determination of proteins in dilute solutions. *Biochim. Biophys. Acta*, **45**, 382–384.
16. H.P. Misra and I. Fridovich (1977) Purification and properties of superoxide dismutase from a red alga, *Porphyridium cruentum*. *J. Biol. Chem.*, **252**, 6421–6423.
17. F. Yamakura (1976) Purification, crystallization and properties of iron-containing superoxide dismutase from *Pseudomonas ovalis*. *Biochim. Biophys. Acta*, **422**, 280–294.
18. J. Kwiatowski, A. Safianowska and Z. Kaniuga (1985) Isolation and characterization of an iron-containing superoxide dismutase from tomato leaves, *Lycopersicon esculentum*. *Eur. J. Biochem.*, **146**, 459–466.
19. M.L. Salin and S.M. Bridges (1980) Isolation and characterization of an iron-containing superoxide dismutase from a eucaryote, *Brassica campestris*. *Arch. Biochem. Biophys.*, **201**, 369–374.
20. M.L. Salin and S.M. Bridges (1982) Isolation and characterization of an iron-containing superoxide dismutase from water lily, *Nuphar luteum*. *Plant Physiol.*, **69**, 161–165.
21. M.V. Duke and M.L. Salin (1985) Purification and characterization of an iron-containing superoxide dismutase from a eucaryote, Ginkgo biloba. *Arch. Biochem. Biophys.*, **243**, 305–314.
22. F.J. Yost, Jr. and I. Fridovich (1973) An iron-containing superoxide dismutase from *Escherichia coli*. *J. Biol. Chem.*, **248**, 4905–4908.
23. N.L. Trant, S.R. Meshnick, K. Kitchener, J.W. Eaton and A. Cerami (1983) Iron-containing superoxide dismutase from *Crithidia fasciculata*. Purification, characterization and similarity to leishmanial and trypanosomal enzymes. *J. Biol. Chem.*, **258**, 125–130.
24. S. Kanematsu and K. Asada (1979) Ferric and manganic superoxide dismutases in *Euglena gracilis*. *Arch. Biochem. Biophys.*, **195**, 535–545.
25. K. Asada and M. Takahashi (1987) Production and scavenging of active oxygen in photosynthesis. In *Photoinhibition* (eds. D.J. Kyle, C.B. Osmond and C.J. Arntzen), Elsevier Science Publishers B.V., Amsterdam, New York, Oxford, pp. 227–287
26. J.M. Palma, L.M. Sandalio and L.A. del Río (1986) Manganese superoxide dismutase and higher plant chloroplasts: A reappraisal of a controverted cellular localization. *J. Plant Physiol.*, **125**, 427–439.
27. J.A. Baum and J.G. Scandalios (1979) Developmental expression and *intracellular localization* of superoxide dismutases in maize. *Differentiation, 13*, 133–140.
28. M.L. Salin and S.M. Bridges (1981) Absence of the iron-containing superoxide dismutase in mitochondria from mustard (Brassica *campestris*). *Biochem. J.*, **195**, 229–233.
29. L.M. Sandalio and L.A. del Río (1987) Localization of superoxide dismutase in glyoxysomes from *Citrullus vulgaris*. Functional implications in cellular metabolism. *J. Plant Physiol.*, **127**, 395–409.
30. M.L. Salin (1988) Toxic oxygen species and protective system of the chloroplast. *Physiol. Plant.*, **72**, 681–689.
31. H.D. Rabinowitch and I. Fridovich (1983) Superoxide radicals, superoxide dismutases and oxygen toxicity in plants. *Photochem. Photobiol.*, **37**, 679–690.
32. L.M. Sandalio, J.M. Palma and L.A. del Río (1987) Localization of manganese superoxide dismutase in peroxisomes isolated from *Pisum sativum* L. *Plant Sci.*, **51**, 1–8.
33. M.J. Droillard (1989) Rôle joué par les radicaux libres et les superoxyde dismutases au cours de la sénescence du pétale d'oeillet coupé (*Dianthus caryophyllus* L., cv. Ember). *Ph.D. Thesis*, Paris VI University, Paris.
34. L.A. del Río, V.M. Fernández, F.L. Rupérez, L.M. Sandalio and J.M. Palma (1989) NADH induces the generation of superoxide radicals in leaf peroxisomes. *Plant Physiol.*, **89**, 728–731.
35. L.M. Sandalio, V.M. Fernández, F.L. Rupérez and L.A. del Río (1988) Superoxide free radicals are produced in glyoxysomes. *Plant Physiol.*, **87**, 1–4.
36. L.A. del Río, F. Sevilla, L.M. Sandalio and J.M. Palma (1989) Nutritional effect and expression of

SODs: Induction and gene expression; diagnostics; prospective protection against oxygen toxicity. In *Fifth Conference on Superoxide and Superoxide Dismutase* (Jerusalem). Abstracts, p. 59.
37. J.M. Palma, M. Gómez, J. Yáñez and L.A. del Rio (1987) Increased levels of peroxisomal active oxygen-related enzymes in copper-tolerant pea plants. *Plant Physiol.*, **85**, 570–574.
38. M. Barón and G. Sandmann (1988) Activities of Cu-containing proteins in Cu-depleted pea leaves. *Physiol. Plant.*, **72**, 801–806.
39. E.F. Elstner (1987) Metabolism of activated oxygen species. In *The biochemistry of plants*, vol. 11 (ed. D.D. Davies), Academic Press, New York, pp. 253–315.
40. F. Sevilla, E. Hellín, C.F. Alcaraz and L.A. del Río (1987) Induction of iron and manganese superoxide dismutases in *Citrus limonum* leaves by iron. *Life Sci. Adv.*, Ser. B., **6**, 133–139.
41. G.L. Matters and J.G. Scandalios (1986) Effect of the free radical-generating herbicide paraquat on the expression of the superoxide dismutase (SOD) genes in maize. *Biochim. Biophys. Acta*, **882**, 29–38.

Accepted by Prof. G. Czapski