Free Rad. Res., Vol. 26, pp. 187–194
Reprints available directly from the publisher
Photocopying permitted by license only

Immunocytochemical Localization of Copper,Zinc Superoxide Dismutase in Peroxisomes from Watermelon (*Citrullus vulgaris* Schrad.) Cotyledons

LUISA M. SANDALIO*, EDUARDO LÓPEZ-HUERTAS, PABLO BUENO and LUIS A. DEL RÍO

Departamento de Bioquímica, Biología Celular y Molecular de Plantas. Estación Experimental del Zaidín, CSIC, Apdo. 419, E-18080 Granada, Spain

Accepted by Professor C. Rice Evans

(Received 9 May 1996; Revised 28 August 1996)

In previous works using cell fractionation methods we demonstrated the presence of a Cu,Zn-containing superoxide dismutase in peroxisomes from watermelon cotyledons. In this work, this intracellular localization was evaluated by using western blot and EM immunocytochemical analysis with a polyclonal antibody against peroxisomal Cu,Zn-SOD II from watermelon cotyledons. In crude extracts from 6-day old cotyledons, analysis by western blot showed two cross-reactivity bands which belonged to the isozymes Cu,Zn-SOD I and Cu,Zn-SOD II. In peroxisomes purified by sucrose density-gradient centrifugation only one cross-reactivity band was found in the peroxisomal matrix which corresponded to the isozyme Cu,Zn-SOD II. When SOD activity was assayed in purified peroxisomes two isozymes were detected, Cu,Zn-SOD II in the matrix, and a Mn-SOD in the membrane fraction which was removed by sodium carbonate washing. EM immunocytochemistry of Cu,Zn-SOD on sections of 6-day old cotyledons, showed that gold label was mainly localized over plastids and also in peroxisomes and the cytosol, whereas mitochondria did not label for Cu,Zn-SOD.

Keywords: Cu,Zn-SOD, cotyledon, immunocytochemistry, peroxisome, watermelon, *Citrullus vulgaris*

INTRODUCTION

Superoxide dismutases (EC 1.15.1.1) are a family of metalloenzymes that catalyze the disproportionation of superoxide ($O_2^{\cdot-}$) radicals, and play an important role in protecting cells against the toxic effects of superoxide radicals produced in different cellular compartments.[1,2] These enzymes generally occur in three different molecular forms containing either Fe, Mn, or Cu plus Zn as prosthetic metals.[2] In higher plants, Cu,Zn-SODs have been reported to be present mainly in chloroplasts and also in the cytosol and mitochondria.[1,3,4] Mn-SODs are chiefly located in mitochondria, and Fe-SODs generally occur in chloroplasts.[1,2,3,4] However, in recent years the presence of different types of SOD in peroxisomes from several plant species have been demonstrated.[3–8] Peroxisomes are subcellular respiratory organelles with an

*Corresponding author. Tel.: 34 58 121011. Fax: 34 58 129600. E-mail: lmsanda@eez.csic.es.

essentially oxidative type of metabolism and have the potential to carry out different metabolic pathways depending on their source.[9] In peroxisomes from pea leaves, by using immunocytochemical and cell fractionation methods a Mn-SOD was localized in the organellar matrix.[4,5] The presence of SOD was also demonstrated in peroxisomes from watermelon cotyledons purified by sucrose density-gradient centrifugation.[3] In this case, two SOD isoenzymes were detected, a Cu,Zn-SOD localized in the matrix, and a Mn-SOD which was apparently bound to the external side of the peroxisomal membrane.[10] The presence of SOD isozymes in peroxisomes has also been reported in organelles purified from cucumber, cotton and sunflower cotyledons,[7] castor bean endosperm,[6] and carnation petals.[8] In recent years, the presence of SOD in plant peroxisomes has been extended to human and animal cells. Peroxisomes from human fibroblasts and hepatoma cells were found to contain Cu,Zn-SOD,[11,12] and the ocurrence of this SOD was also demonstrated in rat liver peroxisomes.[13–15] The generation of $O_2^{\cdot-}$ radicals, the substrate of SOD, in matrices and membranes of plant peroxisomes has been reported and the generating systems of superoxide radicals have been partially characterized.[16–18] Very recently, a Cu,Zn-SOD from peroxisomal origin has been characterized biochemically and immunochemically from watermelon cotyledons.[19,20] The antibody obtained against this isozyme cross-reacted with Cu,Zn-SODs from different plant species which is indicative of the high conservative degree of these proteins.[20]

In spite of the increasingly growing evidence indicating the presence of Cu,Zn-SOD in peroxisomes from plant, human, and animal origin,[3,7,10–15] some doubts have been cast on the peroxisomal localization of plant SODs obtained by cell fractionation methods.[21] These reservations appear to stem from the lack of information on the cDNA(s) encoding peroxisomal Cu,Zn-SODs and the PTS at the COOH-terminus of these enzymes. For this reason, a different experimental approach to the cell fractionation methods employed so far is needed to demonstrate unequivocally the peroxisomal locus of Cu,Zn-SOD in plants. In this respect, monospecific antibodies and immunocytochemical procedures have been applied succesfully in recent years to ellucidate numerous cases of conflicting cellular localizations.

The certainty of the presence of Cu,Zn-SOD in peroxisomes is important to study the function of this SOD in these oxidative cell organelles and also to get deeper insights into the role of peroxisomes in active oxygen-mediated oxidative stress situations induced by biotic and xenobiotic agents. In this work, by using EM immunocytochemistry and western blot analysis in watermelon cotyledons we demonstrate and confirm the presence of Cu,Zn-SOD in a class of plant peroxisomes.

MATERIALS AND METHODS

Plant Material

Seeds of watermelon (*Citrullus vulgaris* Schrad., cv Sugar Baby) were obtained from Fitó (Barcelona, Spain). Seeds were decoated and surface-sterilized with 1% bleach solution for 30 seconds. After several washings, seeds were germinated at 30°C under dark conditions in large Petri dishes on filter paper soaked in distilled water. Cotyledons (first leaves) from 6 day old seedlings were used for the subsequent assays.

Preparation of Tissue Extracts

Excised cotyledons were blended at 0–4°C in 0.1 M Tris-HCl (pH 8.0), 0.1 mM EDTA, 2 mM DTT, 0.2% (v/v) Triton X-100, using a Sorvall Omnimixer (pair cotyledons to medium volume ratio: 2/1). The homogenate was centrifuged at 34,000 g for 30 min at 4°C and the supernatant was recovered.

Isolation of Cell Organelles

Organelles were isolated from 6-d old cotyledons by differential and sucrose density-gradient centrifugation (20–57%; w/w) as described previously.[3] Organelles were recovered from the gradients by puncture with a syringe, and matrix and membrane fractions of peroxisomes were separated by osmotic shock and ultracentrifugation as described.[10] Peroxisomal membranes were purified by treatment with 0.1 M sodium carbonate (pH 11.5) as previously described.[18]

Gel Electrophoresis and Western Blot Analysis

Protein extracts were analyzed by SDS-PAGE in 12% polyacrylamide gels according to Laemmli[22] in a Bio-Rad Mini-Protean II slab cell. For western blot analysis, proteins were electroblotted onto Immobilon nitrocellulose sheets from Millipore (Bedford, MA) and probed with anti-peroxisomal Cu,Zn-SOD as the primary antibody[20] (1/600 dilution) and goat anti-rabbit IgG with horseradish peroxidase (Bio-Rad, Richmond, CA) as the second antibody. Reactivity was detected by a quimioluminiscence method using luminol.[23].

Native PAGE was carried out in 10.5% polyacrylamide gels and SOD activity was localized on the gels by the photochemical NBT stain of Beauchamp and Fridovich.[24]

Electron Microscopy

For electron microscopy, 6-d old cotyledons were collected and processed as described by Corpas *et al.*[25] Pieces were fixed in 0.2% (v/v) glutaraldehyde and 4% (w/v) paraformaldehyde in 50 mM PIPES-KOH buffer (pH 7.4), for 1 h at 4°C. The segments were dehydrated through a graded ethanol series (30–100%; v/v) and infiltrated in LR White resin. The embedded segments were copolymerized at –20°C.

Immunolabeling

Gold sections were mounted on nickel grids and were incubated for 1 h in a blocking solution composed by 2% (w/v) BSA and 5% (v/v) GNS in TBST buffer. TBST buffer consisted of 10 mM Tris-HCl buffer (pH 7.6), 0.9% (w/v) NaCl, 0.05% (v/v) Tween 20, and 0.02% (w/v) NaN_3 (TBST). The sections were then incubated overnight with anti-peroxisomal Cu,Zn-SOD[20] diluted 1:500 in TBST containing 2% (w/v) BSA and 1% (v/v) GNS. Preimmune serum was used as a control. Grids were washed in several drops of TBST and incubated for 1 h in goat anti-rabbit IgG conjugated to 15-nm gold particles (Bio Cell) diluted 1:40 in TBST plus 2% (w/v) BSA. Finally, grids were washed with TBST and distilled water. Sections were poststained in 2% aqueous uranyl acetate for 3 min and examined in a Zeis EM 10C electron microscope.

Other Assays

Proteins were determined with Coomassie blue,[26] using BSA as standard.

RESULTS AND DISCUSSION

In previous works carried out in our laboratory we demonstrated the presence of four different SOD isozymes in extracts of watermelon cotyledons, which were characterized as 2 Mn-SODs (I and II) and 2 Cu,Zn-SODs (I and II).[3] The isozyme Cu,Zn-SOD I has recently been purified and characterized[27] and has very similar molecular properties to peroxisomal Cu,Zn-SOD. The time-course of SOD activity in this tissue showed the appearance of isozyme Cu,Zn-SOD II three days after the beginning of germination whereas the other isozymes were already present in 24 h seeds.[3] The isolation of cell organelles from watermelon cotyledons by sucrose density-gradient centrifugation revealed that Cu,Zn-SOD I was mainly cytosolic and Cu,Zn-SOD II was pre-

sent in the peroxisomal matrix, whereas a Mn-SOD isozyme was located in the external side of the peroxisomal membrane.[10] On the basis of these results we selected cotyledons from 6-d old seedlings to carry out immunological studies to probe the peroxisomal location of the isozyme Cu,Zn-SOD II.

Prior to the immunocytochemical work, the presence of Cu,Zn-SOD in crude extracts and peroxisomal fractions from watermelon cotyledons was studied by specific SOD activity staining and western blotting, using an antibody prepared against the isozyme Cu,Zn-SOD II from watermelon cotyledons.[20] Figure 1 shows the western blot analysis of cotyledon extracts from 6-d old seedlings with the antibody against Cu,Zn-SOD II. In these conditions, two close immunoreactive bands of 16.3 and 16.5 kDa were detected which belonged to the Cu,Zn-SODs I and II, respectively, to judge by the controls with the purified enzymes. However, in the peroxisomal matrix only one band corresponding to the isozyme Cu,Zn-SOD II was detected.

When proteins from peroxisomal fractions (matrices and membranes) were subjected to non-denaturing PAGE and stained for SOD activity, two isozymes were detected, the Cu,Zn-SOD II in the matrix and the Mn-SOD in the membrane fraction (Fig. 2). This latter isozyme was removed by sodium carbonate washing which is indicative that the peroxisomal Mn-SOD in watermelon cotyledons is not an integral membrane protein but a peripheral membrane protein. Similar results have been reported in peroxisomes from castor bean endosperm where there is a Mn-SOD associated to the peroxisomal membrane which is also removed by washing with sodium carbonate.[6]

On immunolabeled sections of cotyledons, gold label was mainly localized over plastids with 18.28 ± 1.28 gold particles per organelle and, to a lesser extent, in peroxisomes where the mean labeling was 3.5 ± 0.43 gold particles per organelle (Fig. 3). Some gold particles were also detected in the cytosol (Fig. 3). On the contrary, no label was observed in mitochondria. In sections probed with preimmune serum no label

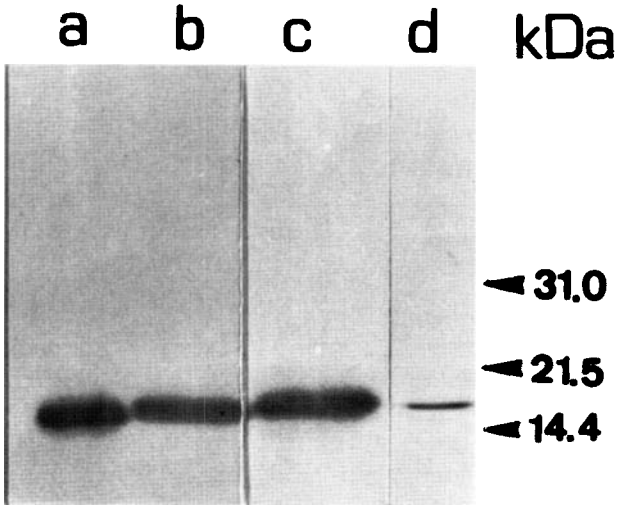

FIGURE 1 Western blot analysis of Cu,Zn-SODs, cell-free extracts, and peroxisomes from watermelon cotyledons with the peroxisomal Cu,Zn-SOD antibody. After SDS-PAGE on 12% polyacrylamide gels, proteins were transfered onto nitrocellulose sheets. Proteins were detected by incubation with a 1/600 dilution of the peroxisomal Cu,Zn-SOD antibody, followed by the second antibody, and staining with luminol. a, purified Cu,Zn-SOD I from watermelon cotyledons[27] (1 μg); b, cell-free extracts from 6-d old watermelon cotyledons (26 μg); c, purified Cu,Zn-SOD II[20] (0.7 μg); d, peroxisomal matrix from watermelon cotyledons (5 μg).

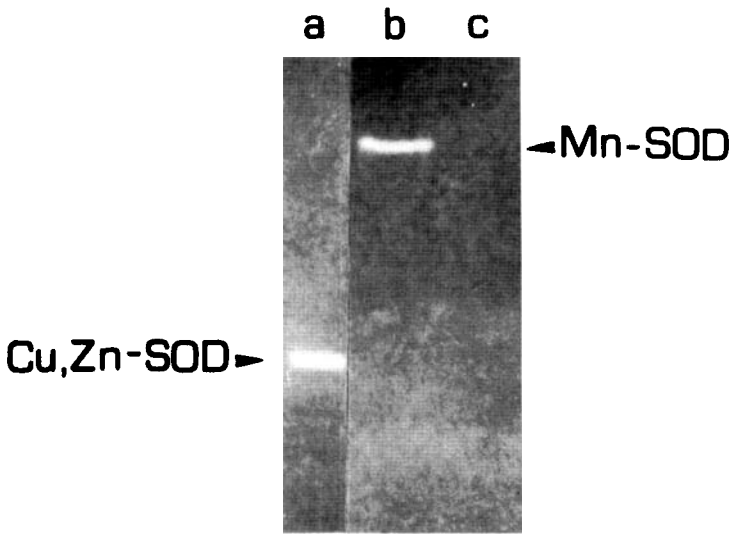

FIGURE 2 Superoxide dismutase activity in peroxisomal fractions. Peroxisomes were purified by sucrose density-gradient centrifugation and proteins were separated by non-denaturing PAGE in a 10% polyacrylamide gel. SOD activity was detected by NBT staining. a, peroxisomal matrices (10 μg); b, peroxisomal membranes (5 μg); c, peroxisomal membranes (5 μg) after washing with 0.1 M sodium carbonate.

was found except in protein and lipid bodies. The labeling detected in the cytosol could be due to the cytosolic Cu,Zn-SOD I isoenzyme but the possibility that part of the labeling could arise from the peroxisomal and plastid forms being imported into these organelles cannot be excluded. These results are the first direct evidence of the localization of Cu,Zn-SOD in peroxisomes from plant tissue, and confirm our previous biochemical studies which demonstrated this cellular locus for Cu,Zn-SOD II. Considering the biochemical and immunological data available, we can finally rule out the possibility of an erroneous localization of Cu,Zn-SOD in peroxisomes due to cross-contaminations or nonspecific adsorption by SODs from other cell compartments.

Recently, the peroxisomal Cu,Zn-SOD from watermelon cotyledons has been purified and characterized.[19,20] This enzyme shares some similarities with Cu,Zn-SODs from other plants, like a 90% amino acid sequence homology with the chloroplastic Cu,Zn-SODs and a 70% homology with the cytosolic Cu,Zn-SODs.[20] However, the peroxisomal Cu,Zn-SOD has some amino acid replacements that differentiate it from most plant SODs, and has a high thermal stability and resistance to inactivation by hydrogen peroxide.[20] This latter characteristic has been considered as a protective adaptation mechanism of the enzyme to the peroxisomal environment rich in H_2O_2.[20] Bueno *et al.*[20] have proposed that chloroplast and peroxisomal Cu,Zn-SODs could be encoded by the same or closely related genes. The product of an unique gene could be targeted bidirectionally to chloroplasts and peroxisomes, like in the case reported for serine:pyruvate aminotransferase of rat liver which is encoded by a single gene and is expressed in peroxisomes and mitochondria.[28]

In peroxisomes a remarkable progress has been made in recent years in the discovery of several peroxisomal targeting signals responsible for the protein import into peroxisomes.[29–31] However, unlike chloroplasts, mitochondria, and ER, the available PTSs do not explain the protein targeting of all peroxisomal proteins. Although many peroxisomal proteins have PTSs located at the

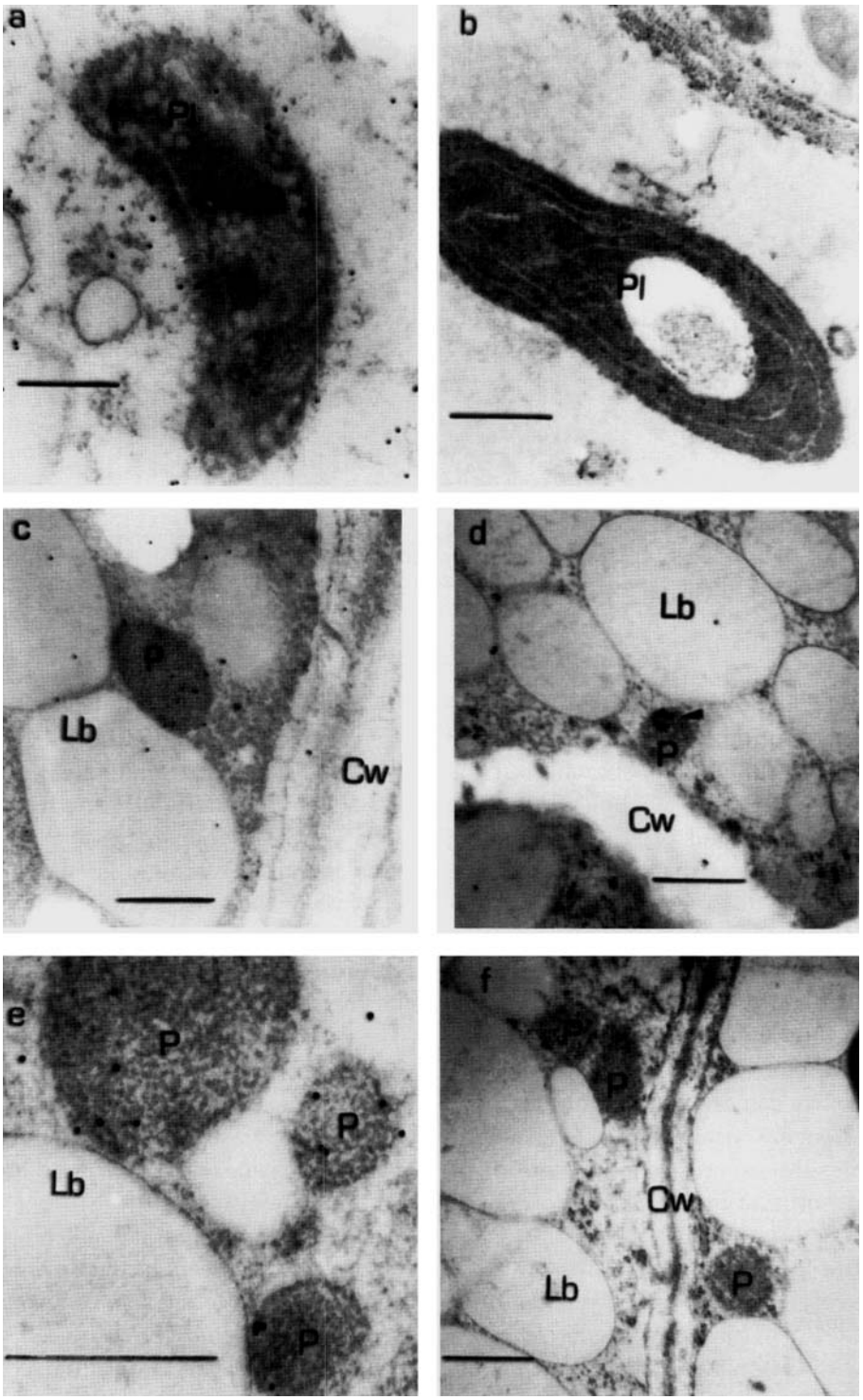

FIGURE 3 Immunocytochemical localization of Cu,Zn-SOD II in cotyledons from 6-d old watermelon seedlings. Cu,Zn-SOD label is present in plastids, peroxisomes and cytosol. a, c, d, and e: sections incubated with Cu,Zn-SOD II antibody (1/500 dilution); b,f: pre-immune serum control. Cw = cell wall; Lb = lipid body; P = peroxisome; Pl = plastid. Arrow in (d) indicates a small peroxisomal area showing Cu,Zn-SOD II label. Bars = 0.5-μm.

COOH-terminus and some at the amino-terminus,[29,30,32] there are also cases of characteristic peroxisomal enzymes, like some catalases among others, which lack it,[29,31,33] and of many non-peroxisomal proteins that contain the consensus PTS within their sequence.[32] As indicated by Baker,[34] the general principles of peroxisomal protein targeting are only gradually emerging and it is likely that actual ideas on peroxisomal protein targeting are naive. Therefore, at present, as far as peroxisomes is concerned, EM immunocytochemical methods appear to be more direct and reliable to prove unequivocally the peroxisomal location of SODs than molecular biology methods based on the isolation of SOD cDNAs containing the necessary targeting sequences, contrary to what has been postulated by other authors.[21,35,36]

The presence of SOD activity in peroxisomes makes sense considering the specialization of this kind of organelles with an essentially oxidative type of metabolism. In the last years we have demonstrated the production of $O_2^{\cdot-}$ radicals in matrices and membranes from plant peroxisomes.[16] In the matrix, the main superoxide producer is xanthine oxidase while in the membrane a small NADH-dependent electron transport chain is involved.[16] More recently, the $O_2^{\cdot-}$-production in peroxisomal membranes from castor bean endosperm has also been demonstrated, as well as the presence of a Mn-SOD in these membranes.[6] In this way, the main role of Cu,Zn-SOD in peroxisomes could be the protection of these cell organelles against $O_2^{\cdot-}$ radicals generated therein. For example, catalase, which is abundant in peroxisomes, is inhibited by $O_2^{\cdot-}$ radicals[37] and Cu,Zn-SOD is inhibited by H_2O_2. Accordingly, Cu,Zn-SOD and catalase could collaborate to protect each other and also to protect peroxisomes by removing $O_2^{\cdot-}$ radicals and H_2O_2, respectively, and so avoiding the possible formation of the vastly more reactive hydroxyl radicals (·OH).[1] This strong oxidizing species damages biological membranes and reacts with most of the compounds present in biological systems, including DNA.[1]

Acknowledgments

The authors acknowledge the help of the Technical Services of the University of Granada for the EM analysis. ELH was recipient of a Ph. D. fellowship (PFPI) from the Ministry of Education and Science, Spain. This work was supported by grant PB92-0492-01 from the DGICYT (Spain), and by grant CHRX-CT93-0184 from the European Union. Financial support from the Junta de Andalucía (Research group No 3315), Spain, is acknowledged.

References

[1] B. Halliwell and J. M. C. Gutteridge (1989). *Free Radicals in Biology and Medicine*, 2nd ed. Clarendon Press, Oxford.
[2] I. Fridovich (1986). Superoxide dismutases. In *Advances in Enzymology and Related Areas of Molecular Biology* (ed. A. Meister) John Wiley & Sons, New York, pp 61–97.
[3] L. M. Sandalio and L. A. del Río (1987). Localization of superoxide dismutase in glyoxysomes from *Citrullus vulgaris*. Functional implications in cellular metabolism. *Journal of Plant Physiology*, **127**, 395–409.
[4] 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 Science*, **51**, 1–8.
[5] L. A. del Río, D. S. Lyon, I. Olah, B. Glick and M. L. Salin (1983). Immunocytochemical evidence for a peroxisomal localization of manganese superoxide dismutase in leaf protoplasts from a higher plant. *Planta*, **158**, 216–224.
[6] L. A. del Río and Donaldson R. P. (1995). Production of superoxide radicals in glyoxysomal membranes from castor bean endosperm. *Journal of Plant Physiology*, **146**, 283–287.
[7] F. J. Corpas, J. Bunkelman, L. Jiang and R. N. Trelease (1994). Identification of superoxide dismutase isoforms in glyoxysomes of oilseeds. *Plant Physiology*, **105**, 146.
[8] M. J. Droillard and A. Paulin, (1990). Isozymes of superoxide dismutase in mitochondria and peroxisomes isolated from petals of carnation (*Dianthus caryophyllus*) during senescence. *Plant Physiology*, **94**, 1187–1192.
[9] A. H. C. Huang, R. N. Trelease and T. S. Moore (1983). *Plant peroxisomes*. Academic Press, New York.
[10] L. M. Sandalio and L. A. del Río (1988). Intraorganellar distribution of superoxide dismutase in plant peroxisomes (glyoxysomes and leaf peroxisomes). *Plant Physiology*, **88**, 1215–1218.
[11] G. A. Keller, T. G. Warner, K. S. Steiner and R. A. Hallewell (1991). Cu,Zn superoxide dismutase is a peroxisomal enzyme in human fibroblasts and hepatoma cells. *Proceedings of the National Academy of Sciences of the United States of America*, **88**, 7381–7385.
[12] J. D. Crapo, T. Oury, C. Rabouille, J. W. Slot and L.-Y. Chang (1992). Copper,zinc superoxide dismutase is primarily a cytosolic protein in human cells. *Proceedings of*

the National Academy of Sciences of the United States of America, **89**, 10405–10409.

[13] I. Singh, G. S. Dhaunsi, J. K. Orak, P. R. Rajagopalan and A. K. Sing (1994). CuZn superoxide dismutase: Intraorganellar distribution in peroxisomes. *Annals of the New York Academy of Sciences*, **723**, 406–408.

[14] G. S. Dhaunsi, S. Gulati, A. K. Singh, J. K. Orak, K. Asayama and I. Singh (1992). Demonstration of Cu-Zn superoxide dismutase in rat liver peroxisomes. Biochemical and immunochemical evidence. *Journal of Biological Chemistry*, **267**, 6870–6873.

[15] R. J. A. Wanders and S. Danis (1992). Identification of superoxide dismutase in rat liver peroxisomes. *Biochimica et Biophysica Acta*, **1115**, 259–262.

[16] L. A. del Río, L. M. Sandalio, J. M. Palma, P. Bueno and F. J. Corpas (1992). Metabolism of oxygen radicals in peroxisomes and cellular implications. *Free Radical Biology and Medicine*, **13**, 557–580.

[17] E. López-Huertas, L. M. Sandalio and L. A. del Río (1994). Preliminary study of the superoxide-generating system of membranes from plant peroxisomes. In *Frontiers of Reactive Oxygen Species in Biology and Medicine*. (eds. K. Asada and T. Yosikawa) Elsevier Science B. V. pp 37–38.

[18] E. López-Huertas, L. M. Sandalio and L. A. del Río (1995). Integral membrane polypeptides of pea leaf peroxisomes: Characterization and response to plant stress. *Plant Physiology and Biochemistry*, **33**, 295–302.

[19] P. Bueno and L. A. del Río (1992). Purification and properties of glyoxysomal cuprozinc superoxide dismutase from watermelon cotyledons (*Citrullus vulgaris* Schrad). *Plant Physiology*, **98**, 331–336.

[20] P. Bueno, J. Varela, G. Giménez-Gallego and L. A. del Río (1995). Peroxisomal copper, zinc superoxide dismutase: Characterization of the isoenzyme from watermelon (*Citrullus vulgaris* Schrad.) cotyledons. *Plant Physiology*, **108**, 1151–1160.

[21] C. Bowler, W. Van Camp, M. Van Montagu and D. Inzé (1994). Superoxide dismutase in plants. *Critical Review in Plant Sciences*, **13**, 199–218.

[22] U. K. Laemmli (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature*, **227**, 680–685.

[23] H. J. Leying, I. Huber and M. Hinzpeter (1992). Detection of protein with the BM chemiluminiscence Western blotting system. *Boehringer Manhein Instruction Manual*.

[24] C. O. Beauchamp and I. Fridovich (1971). Superoxide dismutase: Improved assay and an assay applicable to acrylamide gels. *Analytical Biochemistry*, **44**, 276–287.

[25] F. J. Corpas, J. Bunkelmann and R. N. Trelease (1994). Identification and immunochemical characterization of a family of peroxisome membrane proteins (PMPs) in oilseed glyoxysomes. *European Journal of Cell Biology*, **65**, 280–290.

[26] M. A. Bradford (1976). A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**, 248–254.

[27] J. M. Palma, G. M. Pastori, P. Bueno, S. Distefano and L. A. del Rio (1996). Purification and properties of cystolic copper,zinc superoxide dismutase from watermelon (*Citrullus vulgaris* Schrad.) cotyledons. *Free Radical Research*, in press.

[28] T. Oda, T. Fumai and A. Ichigama (1990). Generation from a single gene of two mRNAs that encode the mitochondrial and peroxisomal serine: pyruvate aminotransferase of rat liver. *Journal of Biological Chemistry*, **265**, 7513–7519.

[29] M. J. de Hoop and G. Ab (1992). Import of proteins into peroxisomes and other microbodies. *Biochemical Journal*, **286**, 657–669.

[30] S. Subramany (1993). Protein import into peroxisomes and biogenesis of the organelle. *Annual Review of Cell Biology*, **9**, 445–478.

[31] L. J. Olsen and J. J. Harada (1995). Peroxisomes and their assembly in higher plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, **46**, 123–146.

[32] S. J. Gould, G. A. Keller, N. Hosken, J. Wilkinson and S. Subramany (1989). A conserved tripeptide sorts proteins to peroxisomes. *Journal of Cell Biology*, **108**, 1657–1664.

[33] W. Ni, R. B. Turley and R. N. Trelease (1990). Characterization of a cDNA encoding cottonseed catalase. *Biochimica et Biophysica Acta*, **1049**, 219–222.

[34] A. Baker (1996). Biogenesis of plant peroxisomes. In: *Membranes; specialised functions in plants* (eds. D. Bowles, J. P. Knox and M. Smallwood) BioScientific (USA), pp. 421–440.

[35] S. Streller, S. Krömer and G. Wingsle (1994). Isolation and purification of mitochondrial Mn-superoxide dismutase from the gymnosperm *Pinus sylvestris* L. *Plant and Cell Physiology*, **35**, 859–867.

[36] S. Streller, S. Karpinski, J. E. Hällgren and G. Wingsle (1994). Four cytosolic-type CuZn-superoxide dismutases in germinating seeds of *Pinus sylvestris*. *Physiologia Plantarum*, **92**, 443–450.

[37] Y. Kono and I. Fridovich (1982). Superoxide radical inhibits catalase. *Journal of Biological Chemistry*, **257**, 5751–5754.