

Identification and immunolocalization of superoxide dismutase isoenzymes of olive pollen

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Gametophytic tissues of plants are an area largely neglected in the broad literature on free radical processes in plants. In order to study the mechanisms of protection against oxidative stress in pollen, the presence of the key antioxidative enzyme superoxide dismutase (SOD; EC 1.15.1.1) was investigated. Crude extracts of olive tree (*Olea europaea* L.) pollen were subjected to native PAGE in 10% polyacrylamide gels. The SOD activity staining of gels showed the presence of four isoenzymes. All the SODs were completely inhibited by 2 mM KCN and 5 mM H₂O₂, and therefore belong to the family of CuZn-SODs. Isoelectric focusing (pH 3.5–7) of crude extracts and further detection of SOD activity allowed determination of isoelectric points for the four isoforms, namely 4.60, 4.78, 5.08 and 5.22. The cross-reactivity of pollen extracts with a polyclonal antibody to cytosolic CuZn-SOD from spinach leaves was assayed by western blotting. After SDS-PAGE and immunoblotting, a major polypeptide band of about 16.5 kDa was detected, which is characteristic of the subunit of most CuZn-SODs. Immunocytochemical studies at TEM level using the same antiserum showed that CuZn-SOD was localized in the cytoplasm of both vegetative and generative cells, and also in material adhered to the pollen wall. The olive pollen CuZn-SODs could function in the protection against oxidative stress during pollen development.

Abbreviations – CuZn-SOD, copper,zinc-containing superoxide dismutase; SOD, superoxide dismutase.

Introduction

The mature pollen grain is released into the environment at anther dehiscence. Once released, as well as during its development, the pollen grain remains exposed to a number of damaging agents, including extreme temperatures, pollutants, UV light, microbial pathogens (Hormaza and Herrero 1994). Most of the information available on the mechanisms of pollen response to these factors is derived from the use of specific stress treatments (i.e. thermal stress, starvation) to induce microspore/pollen embryogenesis, which has a capital importance in plant breeding. However, relatively little information is available on mechanisms against oxidative stress during the gametophytic phase of higher plants.

Superoxide dismutases (SODs; EC 1.15.1.1) are a family of metalloenzymes which catalyse the disproportionation of superoxide (O₂⁻) radicals produced in

different cell loci (Fridovich 1986). They are an important part of the cellular defense system against oxidative stress. Enhanced levels of SOD have been correlated with adverse environmental conditions (Foyer et al. 1994). SODs mainly occur in three different molecular forms containing either Mn, Fe, or Cu plus Zn as prosthetic metals (Fridovich 1986). In higher plants, Mn-SODs are mainly present in mitochondria (Sandalo et al. 1987, Bowler et al. 1994), but also occur in different types of peroxisomes (Sandalo et al. 1987, del Río et al. 1996). Fe-SODs are mainly localized in chloroplasts (Salin 1988), but have also been found in mitochondria and peroxisomes (del Río et al. 1996) and in the cytosolic fraction of legume nodules (Becana et al. 1989). CuZn-SODs are chiefly located in chloroplasts (Palma et al. 1986, Salin 1988) and also in the

cytosol (Baum and Scandalios 1981, Sandalio et al. 1987), the extracellular space (Streller and Wingsle 1994), and in peroxisomes of oilseeds (del Rio et al. 1996, Sandalio et al. 1997, Corpas et al. 1998).

Very few studies have been carried out on the presence of SOD in pollen. In the pollen grain of maize, two Mn-SODs and three CuZn-SODs and their transcripts have been identified in the course of anther dehiscence (Acevedo and Scandalios 1990). SOD activity has also been detected in pollen tubes of lily (Tezuka et al. 1997).

Olive (*Olea europaea* L.) is an important crop in Southern Europe. During its development, olive pollen displays a number of significant biological features (Rodríguez-García and Fernández 1987, Alché et al. 1994), including the presence of allergenic proteins that make this pollen to be considered one of the major causes of respiratory allergy in the Mediterranean area (Bousquet et al. 1985, Rodríguez-García et al. 1995). Several enzymatic systems showing high isoenzymatic variability have been successfully used for cultivar identification in olive (Trujillo et al. 1995), but, to our knowledge, no enzymatic activities involved in oxygen radical metabolism have been employed for this purpose.

The aim of this study was to determine whether key antioxidative enzymes, such as superoxide dismutases, are present in the pollen grain of olive at a critical stage like post-anthesis. And in the present work, the identification and immunocytochemical localization of SOD isoenzymes in mature olive pollen are reported.

Materials and methods

Plant material

Mature anthers (near dehiscence) and pollen were obtained from olive trees (*Olea europaea* L. cv. Picual) growing under natural conditions in several areas of the province of Granada (Spain).

Preparation of crude extracts

Olive pollen (1 g) was stirred in 10 ml 0.01 M ammonium bicarbonate, pH 8.0, at 4°C for 8 h. The mixture was centrifuged at 12000 g for 20 min, and the supernatant filtered through a 0.22- μ m membrane (Sartorius AG, Göttingen, Germany) and used for electrophoresis.

Electrophoresis and immunoblot analysis

Non-denaturing PAGE was performed in 10% tube gels according to Davis (1964). Isoelectric focusing was carried out in a Mini-Protean II (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) slab cell using a pH gradient of 3.5 to 7.0, as described by Palma et al. (1997). The following isoelectric point standards (Phar-

macia LKB Biotechnology, Uppsala, Sweden) were used: pepsinogen (pI 2.80), amyloglucosidase (pI 3.50), red-methyl (pI 3.75), glucose oxidase (pI 4.15), soybean trypsin inhibitor (pI 4.55), β -lactoglobulin (pI 5.20), bovine carbonic anhydrase B (pI 5.85), and human carbonic anhydrase B (pI 6.55). SOD activities were localized in gels by nitro blue tetrazolium chloride reduction with O_2^- radicals generated photochemically (Beauchamp and Fridovich 1971). SOD isoenzymes were distinguished by previous incubation of gels in different inhibitor solutions, 2 mM KCN and/or 5 mM H_2O_2 , for 45 min at 25°C. Isoenzyme activity in the gels was quantified by recording the transmittance of gels at 560 nm in a Shimadzu CS-9000 densitometer (Shimadzu, Columbia, MD, USA).

SDS-PAGE was performed in 12% acrylamide gels, as described by Schägger and von Jagow (1987), and polypeptides were transferred onto polyvinylidene difluoride membranes at 100 V for 1.5 h using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories Ltd). For immunodetection, the membranes were probed with an antibody to cytosol CuZn-SOD from spinach leaves (Kanematsu and Asada 1989), diluted 1:200. A goat anti-rabbit IgG-alkaline phosphatase conjugate (Promega Corporation, Madison, WI, USA), diluted 1:2000, was used as secondary antibody, and the colour was developed with the nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate system.

Protein assay

The protein concentration of samples was determined by the method of Bradford (1976) with bovine serum albumin (BSA) as standard, using the Bio-Rad protein assay.

Ultrastructural immunolocalization of SOD

Mature anthers of *O. europaea* were processed as previously described by Rodríguez-García et al. (1995) with slight modifications. The anthers were dissected from floral buds and fixed in 3% (v/v) glutaraldehyde and 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h at room temperature. They were dehydrated in an ethanol series, gradually transferred to propylene oxide and embedded in Epon. Ultrathin sections (80 nm) were obtained with a Reichert-Jung ultramicrotome and transferred to 300-mesh nickel grids, and these were washed with 5% (w/v) BSA, 1% (v/v) normal goat serum in PBS buffer (blocking reagent) for 15 min. This was followed by incubation at 37°C for 2 h with the same antiserum used for immunoblot analysis, diluted 1:25 in blocking reagent. After washing with PBS, the grids were treated with goat anti-rabbit IgG:20 nm gold (Biocell International), diluted 1:25 in PBS, for 1

h. Then, they were washed in PBS, followed by rinsing in double-distilled water, and stained for 15 min with uranyl acetate. The observations were done in a Zeiss EM10C transmission electron microscope. Control sections were treated as described but omitting the CuZn-SOD antiserum.

Results

The SOD activity in crude extracts of olive pollen was analyzed by native PAGE. Representative densitometric scans are shown in Fig. 1. Four SOD isoenzymes were detected (Fig. 1a). Based on inhibition assays with 2 mM KCN (Fig. 1b) and 5 mM H₂O₂ (Fig. 1c), the four SOD bands were identified as CuZn-SODs. They were numbered I to IV in order of increasing mobility in the gel. Using the relative areas under each peak, the relative activities of each CuZn-SOD were calculated as 44, 28, 19 and 9%, respectively. No evidence was found for Mn-SOD or Fe-SOD isoenzymes. The olive pollen crude extracts were also analyzed by isoelectric focusing in a pH gradient of 3.5–7.0. The isoelectric point values determined for these four CuZn-SODs were 4.60, 4.78, 5.08 and 5.22.

Figure 2 illustrates the Coomassie blue-stained SDS polypeptide profile (lane 1) and immunoblot analysis (lane 2) of pollen crude extracts. By SDS-PAGE at least

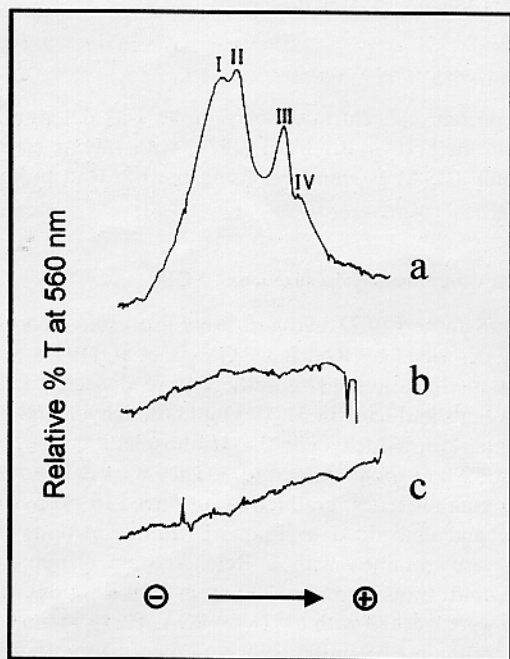


Fig. 1. Isoenzyme activity of SOD in olive pollen crude extracts. Proteins (30 µg) were separated by native polyacrylamide gel electrophoresis. Gels were stained for SOD activity and scanned at 560 nm with a densitometer. a, Gel stained without inhibitors (control). b, Gel stained in the presence of 2 mM KCN. c, Gel stained in the presence of 5 mM H₂O₂.

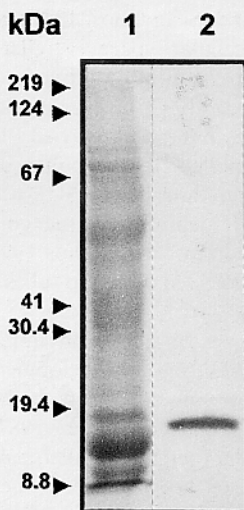


Fig. 2. Western blot of SODs of olive pollen extracts with an antibody to spinach CuZn-SOD. Proteins (30 µg per lane) were separated by SDS-PAGE and transferred onto a PVDF membrane. Lane 1, Coomassie blue staining for proteins. Lane 2, immunoblot probed with anti cytosol-type CuZn-SOD from spinach (1:200 dilution). Molecular mass standards are indicated on the left.

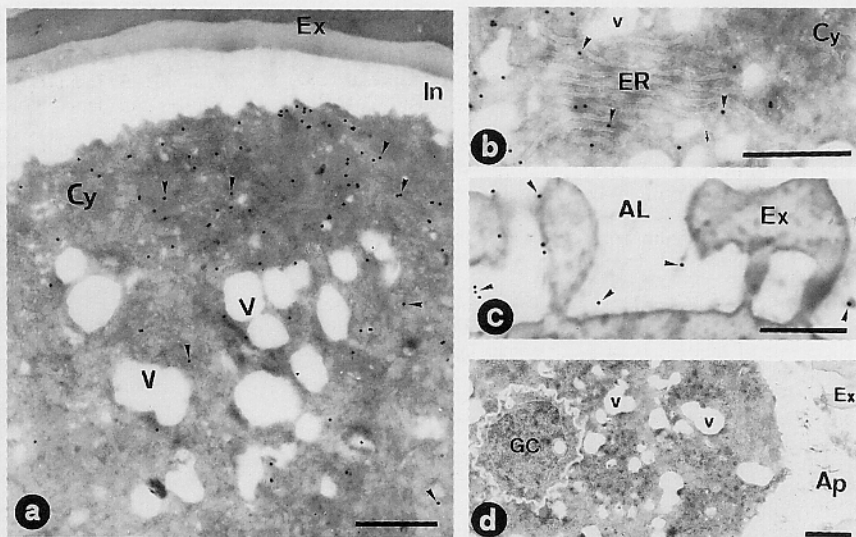
six polypeptides of 72, 53, 18.5, 13.3, 10.2 and 9.3 kDa were distinguished. The polypeptide of 13.3 kDa was the most prominent (lane 1). The antibody against the cytosolic CuZn-SOD from spinach recognized a single polypeptide with a molecular mass of 16.5 kDa (lane 2). This size is in good agreement with the molecular masses described for the CuZn-SOD subunit from other plant species (Kanematsu and Asada 1989, Palma et al. 1997).

The ultrastructural immunolocalization of SOD with the antibody to spinach CuZn-SOD showed that gold particles were mostly in the cytoplasm of both vegetative and generative cells (Fig. 3a). The vegetative and generative nuclei, and the pollen grain wall were not labeled. Gold particles in the cytoplasm were associated with the cytoplasm matrix and the endoplasmic reticulum, while organelles such as mitochondria, plastids, and cytoplasmic vesicles were devoid of gold particles (Fig. 3a,b). Labeling was also detected in material adhered to the outer exine (Fig. 3c). No significant labeling was present in the control (Fig. 3d).

Discussion

The results obtained for SOD activity in nondenaturing polyacrylamide gel electrophoresis and isoelectric focusing indicate the presence of four CuZn-SOD isoenzymes in the mature pollen of olive. Although there are four CuZn-SODs, the immunoblot analysis with the anti-cytoplasmic CuZn-SOD antibody showed a single polypeptide of 16.5 kDa. Identical results were ob-

Fig. 3. Electron micrographs showing the immunocytochemical localization of SOD. Sections of mature pollen grains of *O. europaea* were incubated with antiserum of cytosol-type CuZn-SOD (1:25 dilution). a, Gold particles (arrowheads) are located in the cytoplasm (Cy). Vacuoles (V), exine (Ex) and intine (In) are devoid of gold particles. b, Detail of an *O. europaea* mature pollen grain. Gold particles are concentrated in the endoplasmic reticulum (ER) and cytoplasm. c, Detail of the pollen wall. Material in the outer exine appears labeled (arrowheads). AL, anther locule. d, Section of mature pollen grain incubated with blocking reagent instead of the antiserum (control). Ap, aperture. Bars represent 1 μ m.



tained in western blot assays with the same antibody in sunflower cotyledons, which also have four CuZn-SODs (Corpas et al. 1998). The occurrence of only one type of SOD in olive pollen is not surprising considering that CuZn-SODs are the major isoenzymes in angiosperms and gymnosperms (Kanematsu and Asada 1989). Bridges and Salin (1981), in a survey of the distribution of SOD in vascular plants, also found that a certain number of plant species contained only the CuZn-SOD isoenzymes. Several isoenzymes of superoxide dismutase and catalase have been reported in mature pollen from certain inbred lines of maize (Acevedo and Scandalios 1990), a plant in which SODs do not exhibit any obvious tissue- or stage-specificity during its life cycle.

The ultrastructural localization of SOD in the pollen grain of olive was mainly restricted to the cytosol of both the vegetative and generative cells, as expected from the use of an antiserum raised against a cytosol-type CuZn-SOD. Localization of SOD associated to the material adhered to the exine in the olive pollen grain, reported in this work, points also to the tapetum as a putative source of SODs. One of the functions of the tapetum is the production of sporophytic proteins and enzymes that are deposited onto the pollen surface (Pacini 1994). In several species, these proteins are involved in the process of pollen-stigma recognition (Dickinson and Lewis 1975, Hodgkin et al. 1988) and are localized within the exine.

The presence of SOD in olive pollen grain suggests the existence of a protection mechanism against oxidative stress phenomena, at least during the late stages of

pollen development. This mechanism likely depends on the haploid program of gene expression, although the sporophytic program of the tapetum may also overlap. The preliminary characterization of SODs from olive pollen provides a valuable tool to further investigate changes in their expression levels and activities during pollen development and germination.

Acknowledgments – The authors gratefully acknowledge Drs Sumio Kanematsu and Kozi Asada (The Research Institute for Food Science, Kyoto Univ., Japan) for their gift of antiserum to spinach CuZn-SOD. This work was supported by Project FMBI-CT95-0470 from the European Commission, and by the Junta de Andalucía (research groups CVI 0192 and AGR 160), Spain.

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