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Demonstration of superoxide dismutase enzymes in extracts of pollen and anther of *Zea mays* and in two related products, Baxtin® and Polbax®

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Superoxid dismutase enzymes were isolated and identified in an aqueous extract of pollen and anthers of *Zea mays* and in two commercial products, Baxtin® and Polbax®, derived from the same kind of source material. Prior to analysis the samples were purified by adding soluble poly-N-vinylpyrrolidon and precipitated with ammonium sulphate,

Gel filtration chromatography using a Superose 12 HR column gave well-resolved and similar elution patterns for the maize extract, Baxtin and Polbax samples. Fractions exhibiting superoxide dismutase activity, determined by direct KO_2 assay, were combined, dialysed and evaporated to dryness. The molecular weight of these fractions was approximately 30000 d. These fractions were also analysed by native polyacrylamide gel electrophoresis and stained for superoxide dismutase enzyme activity using nitro blue tetrazolium. The major region of superoxide dismutase enzyme activity was inhibited by addition of cyanide and hydrogen peroxide indicating the presence of a copper, zinc superoxide dismutase. Another minor region of enzyme activity, migrating as standard manganese superoxide dismutase and not inhibited by cyanide or hydrogen peroxide, was also detected.

The results clearly demonstrated the occurrence of both copper, zinc- and manganese superoxide dismutase enzymes in extracts of pollen and anthers of *Zea mays* and also in the two related commercial products, Baxtin and Polbax.

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Active oxygen in biological systems has been thoroughly studied and frequently reviewed (Elstner 1987, Salin 1987, Alscher & Amthor 1988, Leshem 1988, Winston 1990). The harmful effect of active oxygen can be reduced by keeping the O_2-concentration low within the cell through oxidative metabolism or oxygnease reactions. Other possibilities are to keep substrates that produce toxic interme diates separated from each other or by scavenging systems, antioxidants, that reduce the concentrations of active oxygen species. The antioxidants can either be fat-soluble, e.g., carotenoids and ∂-tocopherol, water-soluble, e.g., glutathione and ascorbic acid, or enzymatic compounds, e.g., glutathion reductase and superoxide dismutase (SOD).

SOD is a metalloenzyme or actually a group of metalloenzymes that catalyzes the disproportionation of O_2^-. to yield molecular O_2 and H_2O_2 (Weselake et al. 1986). SOD protects cells against oxygen toxicity by scavenging the superoxide radical and SOD is therefore a key component in the free radical detoxification process. SOD-enzymes have been extensively studied during the last ten years and are now considered present in all aerobic organisms (Rabinowitch & Fridovich 1983, Fridovich 1986, Asada 1988, Monk et al. 1989, Hassan & Scandalios 1990). Three types of SODs have been found in plants, classified according to the metal at the catalytic centre: copper and zinc SOD (CuZn SOD), a manganese-containing homo dimer (Mn SOD) and an iron-containing homo dimer (Fe SOD) (Fridovich 1986). These three types can be distinguished by their sensitivity to inhibitors, CuZn SOD is inhibited by cyanide and H_2O_2 and the Fe SOD is inhibited by H_2O_2, whereas the Mn SOD is insensitive to both inhibitors. Most CuZn SODs isolated so far, with few excptions, are homodimers with a molecular weight of approximately 32.000 (Fridovich 1986). The most abundant SODs in plants are the CuZn SODs, which are found mainly in the cytosol and chloroplasts. Mn SOD is usually localized to the mitochondrial matrix in higher plants (Asada 1988) but has also been isolated from chloroplast thylakoids (Hayakawa et al. 1985). In addition is has been detected in the glyoxysomes (Del Rio et al. 1983, Sandalio & Del Rio 1987).

The development of reproductive structures, e.g., pollen and spores, and the pollination and critical phases in the life cycle of plants. As good protection against the detri-

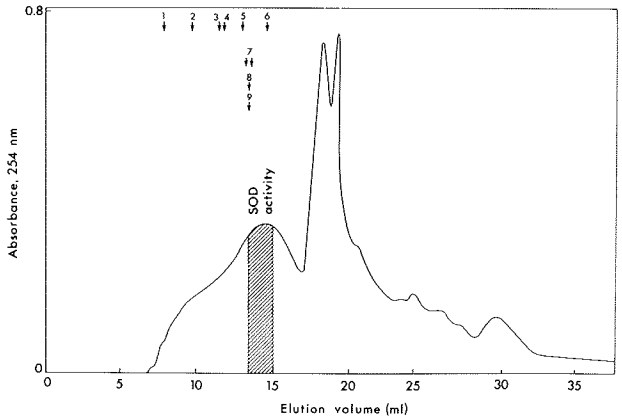

Fig. 1. Superose 12 gel filtration pattern of the maize pollen and anthers extract. The marked area contained SOD activity. Arrows indicate retention volumes of the molecular weight markers and the standard SOD enzymes; thyroglobulin (1), ferritin (2), catalase (3), aldolase (4), ovalbumin (5), chymo–trypsinogen A (6), CuZn SOD (7), Mn SOD (8) and Fe SOD (9).

mental effects of free radicals is therefore necessary to avoid damage to the genetic information. Recently, Acvedo & Scandalios (1990) reported on the expression of SOD genes in mature pollen of maize.

The purpose of the present investigation was to demonstrate the presence of SOD enzymes in three different extracts of pollen and anthers of maize (*Zea mays*); freshly prepared maize extract, Baxtin and Polbax.

MATERIAL AND METHODS

Preparation and purification of maize extract, Baxtin-solution and dissolved Polbax tablets

Fresh pollen and anthers of *Zea mays* (ratio 10:1 w/w) were incubated for 18 h in 200 ml of 50 mM TRIS-HCl, pH 7.5, containing 5 mM $MgCl_2$, 10 mM 2-mercapto-ethanol, 0.4 mM ascorbate, 2 mM EDTA and 4% poly-N-Vinylpyrrolidone 360 (PVP) (w/w). After incubation the sample was homogenized in an ice-bath with an Ultra-Turrax at maximum speed for 5 min. The tissue debris was removed by filtering and the sample was carefully brought to 20% saturation with saturated ammonium sulphate solution, stirred for 30 min and the precipitate was removed by centrifugation at 5000 × g for 30 min. The supernatant was then brought to 90% saturation with addition of solid ammonium sulphate and stirred for 1 h, The precipitate was collected by contrifugation at 5000 × g for 30 min, and dissolved in 2.5 ml of 10 mM TRIS-HCl, pH 7.0 The SOD activity in this extract was tested by the direct KO_2 assay as described below and thereafter isolated using high-performance liquid chromatography equipped with a Superose TM 12 HR 10/30 column (highly cross-linked agarose matrix, Pharmacia LKB Biotechnology, Uppsala, Sweden). The sample was injected off-column via an injector with a 200 µl loop and the column was eluted with 5 mM phosphate buffer containing 0,15 M NaCl at a flow rate of 0.75 ml per min. The absorbance of the eluate was monitored at 254 nm. Forty fractions of 0.75 ml each were collected and the fractions were tested for SOD activity using the direct KO_2 assay.

For calibration of the Superose gel filtration column the elution volumes of the following molecular weight markers was determined; thyroglobulin 669.000 d, ferritin 440.000 d, catalase 232.000 d, aldolase 158.000 d, ovalbumin 43.000 d, chymotrypsinogen A 25.000 d (Pharmacia LKB Biotechnology) and standard SOD enzymes; CuZn SOD from Horseradish, Mn SOD from *E. coli* and Fe SOD from *E. coli* (Sigma Chemical Company, St. Louis, MO, USA).

Baxtin is a commercially available extract solution of fresh and well-controlled pollen and anthers of *Zea mays* (ratio 10:1 w/w) manufactured by Allergon AB, Välinge, Sweden. Ten ml of Baxtin solution (batch no 97808101) was carefully added in drops while stirring to 190 ml of 50 mM TRIS-HCl, pH 7.5, containing 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.4 mM ascorbate, 2 mM EDTA and 4% PVP 360 (w/w). The Baxtin solution was then treated as described above for the freshly prepared maize extract of pollen and anthers.

Polbax (Allergon AB) is a commercially available product produced from a specified amount of grass pollen extract (Pollixin) and Baxtin. Polbax (210 tablets, batch no Q 186218) were ground to a fine powder and dissolved im 200 ml of 50 mM TRIS-HCl, pH 7.5, containing 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.4 mM ascorbate, 2 mM EDTA and 4% PVP 360 (w/w). The Polbax solution was thereafter treated in an analogoua manner to the previously described maize extract.

Determination of SOD activity by the direct KO_2 assay

The SOD enzyme activity in the samples was measured by analyzing the disproportionation of O_2^- according to Marklund (1982). The analysed samples were added in volumes of 20–40 µl to a 1 cm quartz cuvette containing 3 ml of 50 mM 2-amino-2-methyl-1 propanol (AMP)-HCl plus 0.2 mM diethylenetriamine penta acetic acid (DTPA), pH 9.5 and 5 µl 30 µM bovine liver catalase. KO_2 was dissolved in icecold 50 mM NaOH plus 0.5 mM DTPA and 15 µl of this solution was added as substrate to the reaction mixture. The decay of the added O_2^- was then measured continuosly until the baseline had stabilized. KO_2 was added several times to confirm the enzymatic nature of the disproportionation reaction of the analysed samples. The sensitivity of the disproportionation of O_2^- to $CN^-$was also monitored by adding 30 µl of 0.3 M NaCN to the reaction mixture.

Polyacrylamide gel eletrophoresis (PAGE) and NBT staining for SOD activity

Native PAGE was performed at 15°C using PhastSystem and PhastGel homogenous 20 (Pharmacia LKB Biotechnolygy). Pooled fractions containing SOD activity from the gelfiltration chromatography were dialysed for 24 h against 4 l of 5 mM TRIS-HCl buffer pH 7.0 and finally evaporated to dryness. The samples were thereafter dissolved in 10 mM TRIS-HCl buffer, pH 7.0 and applied om native PAGE. The standard SOD enzymes were run in parallel.

The gels were immediately stained for SOD enzyme activity according to Beauchamp & Fridovich (1971) after finished electrophoresis. The gels were first soaked in darkness in 2.45 mM nitro blue tetrazolium (NBT) for 20 min and then soaked in 28 mM tetramethyl ethylene diamine plus 28 µM riboflavin for 15 min. All reagents were dissolved in 36 mM phosphate buffer pH 7.8. The effect of inhibitors in the activity was studied by adding 10 mM NaCN and 10 mM H_2O_2, respectively, to the solutions. After staining in darkness the gels were carefully washed with water and then treated with light. Regions with SOD enzyme activity were then visualized as lighter parts on the darkblue gel.

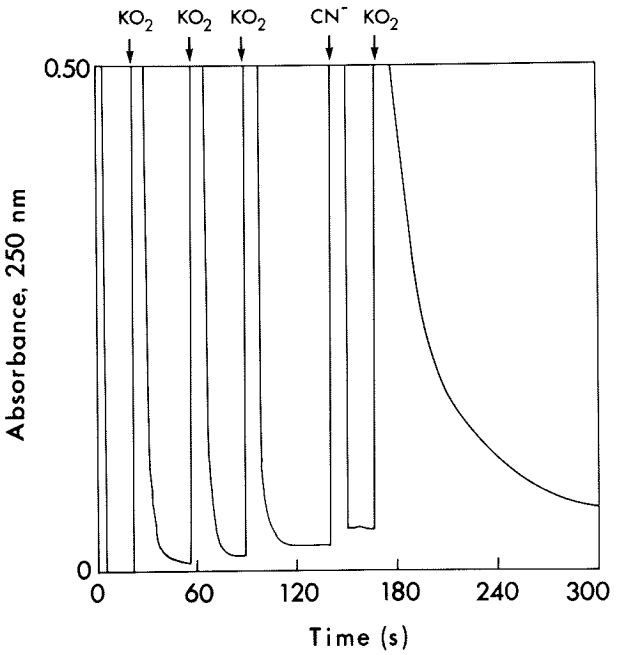

Fig. 2. Disproportionation of $O_2^-\cdot$ by a fraction of the pollen and anthers extract measured spectrophotometrically at 250 nm after various additions of KO_2 and the effect of CN^- on the disproportionation of $O_2^-\cdot$

RESULTS AND DISCUSSION

SOD enzyme activity was measured by the direct KO_2 assay in the crude extract of pollen and anthers of *Zea mays*, Baxtin and dissolved Polbax tablets. Due to analytical disturbances in the samples, however, it was not possible to obtain well-resolved protein bands with defined SOD enzyme activity on native PAGE gels after NBT staining.

Therefore the samples had to be purified to localize the active SOD enzymes in the electrophoretic patterns. The first step in the purification sequence was to absorb interfering compounds, mainly phenols, to soluble PVP. The second step was to apply the maize extract on a Superose 12 HR 10/30 column for further purification by gel filtration chromatography. The gel filtration elution pattern of the pollen and anther extract is shown in Fig. 1. SOD enzyme activity was detected by the direct KO_2 assay in fractions eluting between 13.5 and 15 ml. These active frations were pooled for further analysis. Fig. 2. shows the O_2^- disproportionation activity of the fractions eluted at 14.25–15.00 ml versus time after several additions of KO_2. The repeatability of the activity pattern after KO_2 addition for this maize fraction indicates that the molecule(s) responsible for the disproportionation reaction are not consumed and thus constitute an enzyme molecule. Furthermore the effect of CN^-,a Cu ZnSOD enzyme inhibitor, on the O_2^--disproportionating activity of the maize fraction is also shown in Fig. 2. The maizederived pollen and anther fraction exhibits SOD enzyme activity and the main part of this activity is

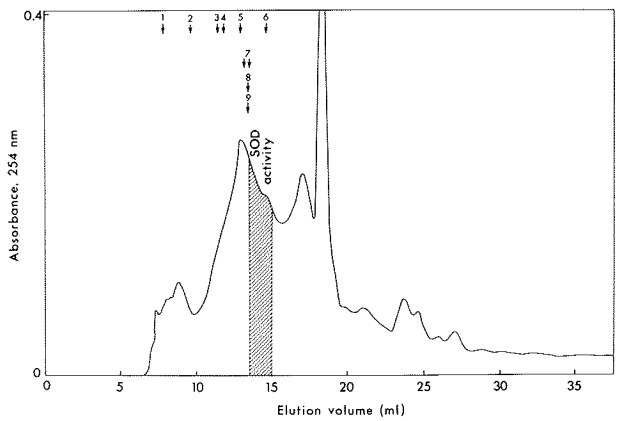

Fig. 3. Superose 12 gel filtration pattern of the Baxtin sample. Experimental conditions as in Fig. 1.

inhibited by the addition of CN^-. The cyanide sensitivity of the maize fraction confirms that the enzyme is copper and zinc-containing SOD (Fridovich 1986).

Fig. 1 also presents data about elution volumes of the molecular weight markers. The identified SOD enzyme activity is located between the protein markers at 25000 d and 43000 d and further the standard SOD enzymes chromatographed in parallel with the maize extract also elutes in this molecular weight region. A linear regression of the logarithmic molecular weights versus retention volumes resulted in a coefficient of determination of 0.97 and an approximate mean molecular weight of the putative SOD enzyme of 28.600.

The gel filtration elution profiles of the purified Baxtin and Polbax samples are shown in Figs. 3 and 4. The Superose elution patterns of Baxtin (Fig. 3) and the freshly prepared maize extract (Fig. 1) are very similar, while the pattern for Polbax differ in the low molecular weight region (Fig. 4), most likely due to the presence of grass pollen components (e.g., Pollixin). However the elution volume of the fractions containing SOD enzyme activity is located in the same region. Both Baxtin and Polbax samples exhib-

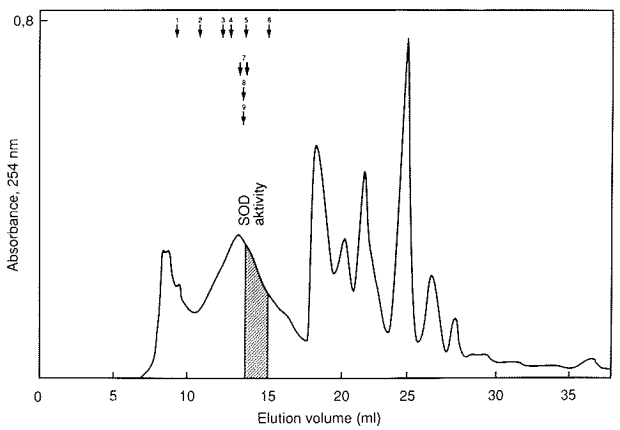

Fig. 4. Superose 12 gel filtration pattern of the Polbax sample. Experimental conditions as in Fig. 1.

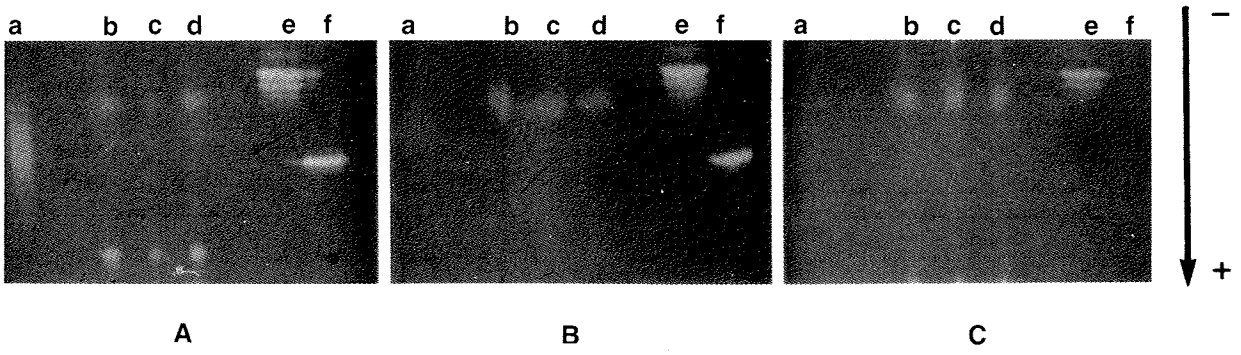

Fig. 5. A–C. Native PAGE gels stained for SOD enzyme activity with NBT after analysis of standard CuZn SOD (500 ng) (a), pollen and anthers extract (b), Baxtin sample (c), Polbax sample (d), standard Mn SOD (250 ng) (e) and standard Fe SOD (250 ng) (f). Polyacrylamide gels; (A) without inhibitor (B) in the presence of 10 mM CN^- during staining and (C) in the presence of 10 mM H_2O_2 during staining.

ited maximum SOD enzyme activity in the fractions eluting between 13.5 ml and 15 ml. The main part of the SOD enzyme activity was also inhibited by the addition of 10 mM CN^- to the reaction medium when performing the direct KO_2 assay, confirming the presence of CuZnSOD in Baxtin and Polbax.

The homogeneity of the SOD enzyme activity in the pooled fraction from Superose gelfiltration was analysed by PAGE. The polyacrylamide gel used allow complex mixtures of proteins to be separated in the molecular weight range 2000–150000 d. Fig. 5 illustrates three native PAGE gel patterns with standard CuZn SOD, FeSOD, MnSOD and the pooled fractions (13.5–15.0 ml) of the freshly prepared maize extract, Baxtin and Polbax samples in the absence and presence of SOD enzyme inhibitors during the staining procedure. The SOD enzyme activity in the various maize samples applied on the gel was developed by using the NBT reagent.

Fig. 5A demonstrates distinct SOD enzyme activity for all three analysed maize samples (lane b,c,d) and further the activity is localised to defined regions on the gel. The enzyme activity of the SOD standards run in parallel is easily localised in the PAGE gel pattern. The enzyme activity of standard CuZn SOD and MnSOD partly overlap the SOD enzyme activity of the maize samples. Fig. 5B shows a PAGE analysis where the gel was treated with 10 mM CN^- and Fig. 5C with 10 mM H_2O_2 during the staining procedure. Inactivation of the enzyme activity (lane b,c,d) in the presence of CN^- confirmed that the SOD activity was due to CuZnSOD (Fig. 5B). The region located closer to the front was clearly inhibited by the addition of CN^- and H_2O_2 as was also the standard CuZn SOD (Fig. 5B and 5C). On the contrary the region close to the application point on the PAGE gel was neither inhibited by CN^- nor H_2O_2 and migrated as standard Mn SOD (lane e), therefore indicating the presence of a Mn SOD in the samples. The activity of the standard Fe SOD (lane f) was inhibited by the addition of H_2O_2 but not by addition of CN^- (Fig. 5B and 5C). No further change in the SOD enzyme activity pattern of maize pollen and anther extract, Baxtin or Polbax samples could be detected after H_2O_2 treatment (Fig. 5C) demonstrating that FeSOD is not present in the analysed maize extracts. The NBT activity staining patterns are in agreement with the gelfiltration data i.e. the detected SOD enzyme activity in the analysed maize preparations are located in the regions for low molecular weight protein molecules

CONCLUSIONS

The presence of superoxide dismutase enzymes in extracts of pollen and anthers of *Zea mays* and in the related products Baxtin and Polbax was established after purification of the samples by a simple PVP precipitaion. Superose gel filtration of the purified samples revealed that SOD enzyme activity was located around 30000 d. The enzyme activity was inhibited by CN^- indicating the presence of CuZnSOD. Polyacrylamide gel electrophoresis followed by NBT activity staining in the absence and presence of different inhibitors confirmed that the SOD enzyme activity in the pooled fractions from Superose gelfiltration of the freshly prepared maize extract, Baxtin and Polbax samples was due to CuZnSOD and MnSOD.

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