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Superoxide synthase and dismutase activity of plasma membranes from maize roots

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Summary. Superoxide synthase and superoxide dismutase activity have been monitored in isolated maize (*Zea mays*) root plasma membranes spectrophotometrically by determination of nitro-blue tetrazolium and cytochrome *c* reduction, respectively. Superoxide production was induced by NADH and NADPH, with similar kinetics and approaching saturation at 0.06 mM in the case of NADPH and 0.1 mM in the case of NADH, with rates of 18.6 ± 5.0 and 21.8 ± 7.2 nmol/min·mg of protein, respectively. These activities exhibited a broad pH optimum between pH 6.5 and 7.5. Diphenylene iodonium inhibited about 25% (10 μM DPI) and 40% (100 μM DPI) of this activity, imidazole inhibited about 20%, while KCN, a peroxidase inhibitor, did not show any significant inhibition. Superoxide-dismutating activity was shown to occur in the same isolates and depended on the quantity of plasma membrane protein present. Growth of plants on salicylic acid prior to membrane isolation induced a rise in the activity of both of the enzymes by 20–35%, suggesting their coordinated action.

Keywords: Plasma membrane; Redox system; Salicylic acid; Superoxide dismutase; Superoxide synthase; *Zea mays*.

Abbreviatons: DPI diphenylene iodonium; SOD superoxide dismutase.

Introduction

The production and removal of the extracellular active oxygen species in plants may be mediated by the activity of enzymes located in the plasma membrane or in the apoplast. Although the real molecular mechanism of H_2O_2 production at the plant cell surface is still unknown, two possible mechanisms have been proposed, one involves the action of a peroxidase (Ros Barcelo 2000) and the other the action of an NAD(P)H oxidase similar to that observed in mammalian neutrophils (Murphy et al. 1998).

Plasma-membrane-bound superoxide synthase (superoxide-generating NAD(P)H-oxidoreductase) has been described in cultured rose cells (Murphy and Auh 1996) and partially purified from bean plasma membrane preparations (van Gestelen et al. 1997). This enzyme is thought to transfer electrons from cytosolic NAD(P)H to oxygen in the apoplast, thus producing superoxide radicals in the apoplast. The formation of superoxide can further result in other reactive oxygen species such as hydrogen peroxide and hydroxyl radical. The superoxide-scavenging enzyme superoxide dismutase (SOD), which catalyzes the disproportionation of superoxide to hydrogen peroxide, has been reported to be present in the apoplast and associated with the sites of superoxide generation in the vascular tissue (Ogawa et al. 1997). Also, high-isoelectric-point isoforms of SOD were found to be localized in the plasma membranes of sieve cells (Karpinska et al. 2001).

In recent years a considerable body of evidence has accumulated suggesting that salicylic acid induces plant resistance to pathogens and other stress factors (Raskin 1992). Although the mechanism of action of salicylic acid is not clear, it was proposed that salicylic acid signaling is mediated by active oxygen species (Kawano and Muto 2000).

The aim of this work was to determine the presence and the characteristics of plasma-membrane-bound superoxide-generating and -dismutating activities in

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maize roots and their dependence on exogenous application of salicylic acid.

Material and methods

Plant material

The inbred line VA35 of maize (*Zea mays* L.) was used. The seeds, germinated for 3 days on water, were grown for 14 days on modified Knopp solution, the concentrations of NO_3^- and NH_4^+ being 10.9 and 7.2 mM, respectively (Hadži-Tašković Šukalović et al. 1999). The plants were grown in a controlled environment under a regime of 12 h light and 12 h dark at 22 and 18 °C, respectively, and relative humidity of 70%. In experiments with salicylic acid, 0.1 mM salicylic acid was added to the nutrition solution 24 h before the experiments.

Plasma membrane isolation

Cut roots were ground in cold grinding buffer (250 mM sucrose, 3 mM EDTA, 50 mM Tris-HCl [pH 7.5], 1 mM dithiothreitol, and 10% [w/v] glycerol) with a chilled mortar and pestle. The homogenate was filtered and centrifuged at 12000 **g** for 10 min. The supernatant was centrifuged at 100000 **g** for 30 min. The microsomal fraction in the pellet was washed with a washing buffer (2 mM Tris-HCl [pH 7.5], 250 mM sucrose, 10% [w/v] glycerol) by resuspending and pelleting by centrifugation at 100000 **g** for 30 min. The washed microsomes were suspended in phase buffer (5 mM K-phosphate buffer [pH 7.8], 330 mM sucrose, 3 mM KCl) for plasma membrane purification by partitioning in a two-phase system. For phase partitioning, microsomes (3 g) were added to make a 12 g two-phase system that contained 6.5% (w/w) dextran T 500, 6.5% (w/v) polyethylene glycol 335, 330 mM sucrose, 3 mM KCl, and 5 mM K_2HPO_4, pH 7.8. The tubes were mixed by inversion and centrifuged in a swinging-bucket rotor for 5 min at 1500 **g**. The upper phase was collected and repartitioned on a fresh lower phase. This procedure was repeated three times and the final upper phase was diluted with 10 volumes of washing buffer without glycerol and centrifuged at 100000 **g** for 30 min. The resulting pellet, containing purified plasma membranes, was resuspended in the same washing buffer to give a final concentration of 3–4 mg of protein per ml and stored at –60 °C. The purity and characterization of such membranes has been described previously (Hadži-Tašković Šukalović et al. 1999). The protein content was measured in the presence of 0.005% Triton X-100 by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Plasma membrane washing

A small volume of purified plasma membrane suspension was diluted 30-fold in 2 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 150 mM KCl, and 0.01% Triton X-100 in order to remove peripheral and adsorbed soluble proteins. The membranes were pelleted at 100000 **g** for 30 min, resuspended in washing buffer without glycerol, pelleted again, and resuspended in a small volume of the same buffer.

Enzyme activities

Superoxide synthase activity was determined as NAD(P)H-dependent O_2^- generation by the reduction of the tetrazolium dye nitroblue tetrazolium to monoformazan by two molecules of O_2^- (van Gestelen et al. 1997). This reduction was detected spectrophotometrically at 530 nm, and the O_2^- concentration (monoformazan concentration) was calculated with an extinction coefficient of 12.8 $mM^{-1}\,cm^{-1}$ (Bielski et al. 1980). The selective reduction of nitroblue tetrazolium by O_2^- was calculated from the difference in the nitroblue tetrazolium reduction rate in the presence and the absence of SOD (100 units/ml). The reaction mixture consisted of 50 mM Tris-HCl buffer, pH 7.5 (except in the case of pH studies where it was varied from pH 5.5–8.0), 300 mM sucrose, 0.1 mM nitroblue tetrazolium, 0.025% (w/v) Triton X-100, and 30–50 µg of protein. The reaction was started by the addition of NADH or NADPH. Superoxide-dismutating activity was determined spectrophotometrically by measuring the inhibition of cytochrome *c* reduction by superoxide anion radicals, generated by the xanthine oxidase-xanthine reaction, at 550 nm (McCord and Fridovich 1969), in the presence of plasma membranes. The reaction mixture consisted of 50 mM K-phosphate buffer, pH 7.8, 0.1 mM EDTA, 0.1 mM cytochrome *c*, 1 mM xanthine, and xanthine oxidase at the concentration needed to obtain an absorbance change of 0.025 per min. All experiments were performed at 30 °C.

The results presented were obtained on at least two replications made on three independent membrane preparations.

Results

Purified plasma membranes generated superoxide as measured by the SOD-sensitive nitroblue tetrazolium reduction. The superoxide synthase activity was induced with both NADH and NADPH. Superoxide synthase activities in washed plasma membranes, determined for 5 plasma membrane preparations, were 22 ± 7 nmol/min·mg of protein induced by 0.1 mM NADH, and 19 ± 5 nmol/min·mg of protein by 0.06 mM NADPH. Superoxide synthase activity was studied in the presence of different concentrations of NADH and NADPH as substrates (Fig. 1). The results obtained showed similar kinetics in either case, with K_m values of 0.019 mM for NADH and 0.02 mM for NADPH. pH dependence of superoxide synthase activity, determined over a pH range from 5.5 to 8.0, exhibited a broad optimum between pH 6.5 and 7.5 (Fig. 2).

The effect of some inhibitors on superoxide generation was investigated in the presence of NADH. KCN, an inhibitor of peroxidase activity used to exclude this activity in superoxide production, at a concentration of 1 mM was almost completely ineffective (for $n = 9$, the specific activity was $96\% \pm 3\%$ compared with the values of controls without inhibitor). Diphenylene iodonium (DPI), an inhibitor of flavoenzymes, inhibited the superoxide synthase activity partially (for $n = 6$ each, the specific activities were $75\% \pm 5\%$ and $59\% \pm 11\%$, at 10 and 100 µM DPI, respectively). Similarly, imidazole, an inhibitor of superoxide synthase activity, at a concentration of

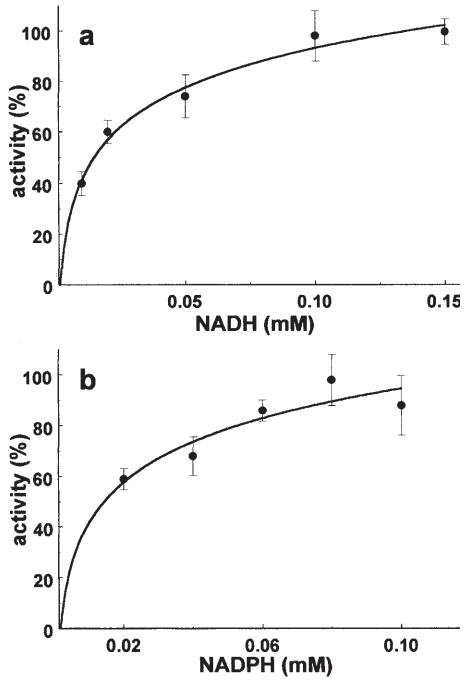

Fig. 1. Activities of maize root plasma membrane superoxide synthase as a function of substrate concentrations, NADH (**a**) and NADPH (**b**), expressed as percentage of the maximal value observed. The error bars represent standard errors, the number of measurements being 6–9

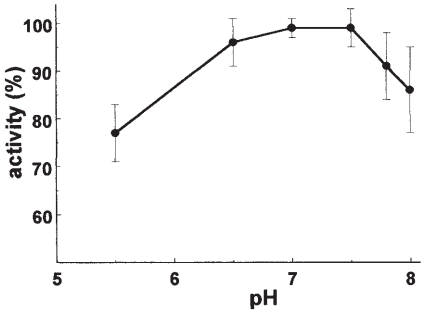

Fig. 2. pH dependence of plasma membrane O_2^- synthase activity, expressed as percentage of the maximal value observed. The measurements were performed in the presence of 0.1 mM NADH. The error bars represent standard errors, the number of measurements being 6–9

10 mM induced an inhibition of superoxide generation to 80% ± 6% (n = 6) in our preparations.

Plasma membrane preparations showed an inhibition of cytochrome *c* reduction induced by superoxide radical produced in xanthine-xanthine oxidase reaction, indicating superoxide-dismutating activity. The rate of cytochrome *c* reduction induced by superoxide radical decreased with an increase in the concentration of plasma membrane protein (Fig. 3).

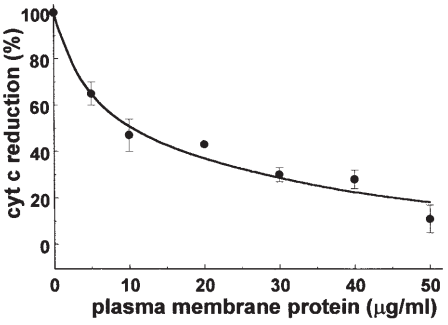

Fig. 3. Cytochrome *c* reduction induced by superoxide radical in the presence of different quantities of plasma membrane protein, expressed as percent of activity obtained in the absence of plasma membranes. The error bars represent standard errors, the number of measurements being 6–9

To ascertain that only plasma-membrane-associated enzymes were studied, but not some loosely bound soluble proteins, the purified plasma membranes were subjected to a salt-washing procedure. Such treatment did not significantly remove either NAD(P)H O_2^- synthase activity (for n = 6 each, the specific activities were 104% ± 10% and 90% ± 9% at 0.1 mM NADH and 0.06 mM NADPH, respectively, compared with the values of unwashed preparations) or superoxide-dismutating activity (95% ± 8%, n = 6).

Both superoxide synthase and superoxide-dismutating activity were stimulated by the presence of salicylic acid in the plant growth medium. The specific activities of superoxide synthase were 134% ± 14% and 123% ± 11% (n = 6 each) at 0.1 and 0.06 mM NADH, respectively, compared with the values of plasma membranes from plants not treated with salicylic acid; the specific SOD activity was 120% ± 9% (n = 6).

Discussion

Superoxide synthase activity has been previously demonstrated with plasma membrane preparations from cultured rose cells (Murphy and Auh 1996), bean seedlings (van Gestelen et al. 1997) and wheat roots (Quartacci et al. 2001). Recently, Sagi and Fluhr (2001) have shown that plasma membranes isolated from tomato and tobacco cells exhibit NADPH-dependent O_2^--producing sites in protein bands separated by polyacrylamide gel electrophoresis, associating this activity with 106, 103, 80 and 75 kDa bands. Our plasma membrane preparations catalyzed the synthesis of superoxide radical in the presence of both NADH and NADPH as electron donors. Such an activity was also demonstrated with both substrates

in the case of plasma membranes from rose cells (Murphy and Auh 1996), although with different affinity. The solubilized enzyme from bean plasma membranes showed however a high preference toward NADPH as substrate (van Gestelen et al. 1997).

DPI has been noted as an inhibitor of superoxide generation in several plant systems. Although the appearance of superoxide in an oxidative burst was blocked by very low concentrations of DPI in rose cells (2 μM needed for 50% inhibition) (Bolwell et al. 1998), much higher concentrations were required for the inhibition of purified superoxide synthase activity from bean plasma membranes (0.1 mM produced 73% inhibition) (van Gestelen et al. 1997). Frahry and Schopfer (1998) obtained a 50% inhibition of peroxide-catalyzed production of H_2O_2 at a much lower DPI concentration (10 μM), which could argue in favor of the peroxidase instead of a NAD(P)H oxidase-type of enzyme being inhibited. In our experiments, 10 μM DPI produced a 25% inhibition, whereas 0.1 mM DPI resulted in about 40% inhibition of the enzyme activity. The second superoxide synthase inhibitor used in our experiments, 10 mM imidazole, inhibited also about 20% of the superoxide synthase activity, while in the case of bean plasma membranes it inhibited about 60% of the activity (van Gestelen et al. 1997). Iizuka et al. (1985) have shown that pyridine and imidazole at higher concentrations (150–200 mM) induced an inhibition of animal neutrophil oxidase activity. In the case of rose cells, 10 μM imidazole induced a 20% inhibition of O_2^- synthase activity when NADH was the substrate (Murphy and Auh 1996).

The insensitivity of superoxide synthase activity to KCN observed in the case of our plasma membranes, as well as the decrease of this activity at lower pH, argues that a peroxidase is not involved in this superoxide production. However, it should be also noted that our membrane preparations show differences with respect to the observed DPI inhibition, when compared to those of other authors.

The superoxide-scavenging enzyme SOD was shown to exist in a number of forms and in practically all of the cellular compartments. SOD in the apoplast appears to function in the biosynthesis of lignin by causing rapid disproportionation of the superoxide anion radical prior to its interaction with cellular components. The localization of SOD in the apoplastic region of spinach leaf and hypocotyl tissues corresponds to that of the accumulation of lignin (Ogawa et al. 1996). Immunogold electron microscopy localized high-isoelectric-point isoforms of SOD in the plasma membranes of sieve cells and Golgi apparatus (Karpinska et al. 2001). Superoxide-dismutating activity of our plasma membranes, which increased with an increase in the concentration of plasma membrane protein, suggests the involvement of plasma membrane enzyme in this activity. Further characterization of this activity is required.

The effect of salicylic acid on both superoxide synthase activity and superoxide-dismutating activity of plasma membrane preparations is in agreement with results showing an increased generation of superoxide and H_2O_2 induced by salicylic acid (Kawano and Muto 2000). Although inhibition of catalase by salicylic acid in vitro was shown (Chen et al. 1993), later results demonstrated that salicylic acid-enhanced H_2O_2 levels were related to increased activities of Cu,Zn-superoxide dismutase (Rao et al. 1997). Our results show a concomitant increase in the activity of both the superoxide synthase and the superoxide-dismutating activity in membranes isolated from plants grown on salicylic acid.

To conclude, isolated plasma membranes of maize roots are capable of generating and scavenging superoxide radicals. Both of these activities were not removed by a washing procedure that removes loosely bound or nonmembranous proteins, confirming their association with the plasma membrane. Their concomitant increase in plants subjected to salicylic acid argues in favor of their correlated action. Since there is evidence for the requirement for active oxygen species in cell growth, extension, and resistance in the apoplast (Olson and Varner 1993, Mehdy 1994), the role of the superoxide synthase that could couple the intracellular reducing environment to the production of reduced oxygen in the extracellular space has a sound physiological role. The simultaneous existence of a superoxide-dismutating activity on the same membranes could in such a case serve as a protective mechanism for some of the susceptible membrane components in a situation where such species are produced and exist in their vicinity.

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