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COPPER-INHIBITED NADH-DEPENDENT PEROXIDASE ACTIVITY OF PURIFIED SOYA BEAN PLASMA MEMBRANES

M. ZANCANI, G. NAGY, A. VIANELLO and F. MACRÌ*

Dipartimento di Biologia ed Economia Agro-industriale, Sezione di Biologia Vegetale, Università di Udine, Via Cotonificio 108, I-33100 Udine, Italy

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Key Word Index—*Glycine max*; Leguminosae; soya bean; peroxidase; plasma membrane; copper.

Abstract—Highly purified soya bean plasma membranes exhibited peroxidase activity. Divalent copper strongly inhibited NADH oxidation (but not H_2O_2 oxidation) catalysed by this enzyme, whereas Cd^{2+}, Ni^{2+} or Zn^{2+} had little or no effect. This inhibition did not depend on a reaction by sulphydryl groups, or on a replacement of ions in the enzyme by Cu^{2+}. The effect of Cu^{2+} may be explained by its scavenging capability towards O_2^- which is produced during NADH-dependent peroxidase activity.

INTRODUCTION

Soils occasionally contain phytotoxic amounts of metals, but more frequently, they accumulate them as a consequence of industrial and agricultural activities [1]. The mechanism by which toxicity occurs, may be differently explained by (i) binding of metals to sulphydryl groups, or (ii) replacement of an essential metal in metallo-proteins or metal–protein complexes [2]. The resistance of plants to metals may depend on biochemical detoxification, compartmentalization of the toxic elements within the cell, or on a limited uptake of metals into cells [3].

Many transition elements are of great biological importance. All are metals and the most important feature is their variable valency which allows them to undergo changes in oxidation state involving one electron. This property enables some transition metals to act as cofactors of metallo-protein enzymes, although, at high concentrations, they can become phytotoxic, probably interfering, in their free state, with oxidoreduction reactions [4].

Several redox systems have been identified in or on the surface of plant cells (cell wall–plasmalemma interface) [5, 6]. Among these is an NADH oxidase/peroxidase (EC 1.11.1.7) acting as a terminal oxidase, associated to plasma membrane or cell wall [7]. This enzyme which contains a ferriprotoporphyrin as prosthetic group is able to oxidize several molecules in the presence of hydrogen peroxide or oxygen. The functions of peroxidases are still obscure but, at least for those localized at the surface of plant cells, their involvement in H_2O_2 synthesis [8–10], cross-linking of cell wall components [11], polymerization of lignin and suberin monomers [12] and response of plants to stress [6] has been established.

The plasmalemma is the first target for toxic ions and, in addition, the maintenance of its integrity may also have a role in tolerance to metals [13]. Therefore, this work was undertaken to verify whether several divalent cations, some of which are transition metals, may interfere with peroxidase activity associated to highly purified soya bean plasma membranes.

RESULTS

Peroxidase activity in isolated soya bean plasma membranes

Peroxidase activity may be evaluated either as NADH oxidation (H_2O_2 formation) or as *o*-dianisidine oxidation (H_2O_2 breakdown) [14]. Figure 1 (A) shows that the addition of NADH to highly purified soya bean plasma membranes induced a decrease of absorbance a 340 nm (NADH oxidation) which was slightly enhanced by salicylhydroxamic acid (SHAM) and strongly stimulated by the subsequent addition of Mn^{2+}. Superoxide dismutase (SOD), catalase, azide and KCN, all completely inhibited NADH oxidation when added during the time-course of the reaction. The apparent K_m for this activity was *ca* 200 μM. The same pattern of responses was obtained following NADH oxidation as oxygen consumption (results not shown). The activity was also followed as *o*-dianisidine oxidation (Fig. 1B). The addition of H_2O_2 to membranes caused a rapid increase of absorbance at 460 nm which was inhibited by azide or KCN. This oxidase was an integral protein, not superficially attached to membranes, because treatment by 0.01% Triton X-100 or 0.7 M NaCl did not cause its release from

*Author to whom correspondence should be addressed.

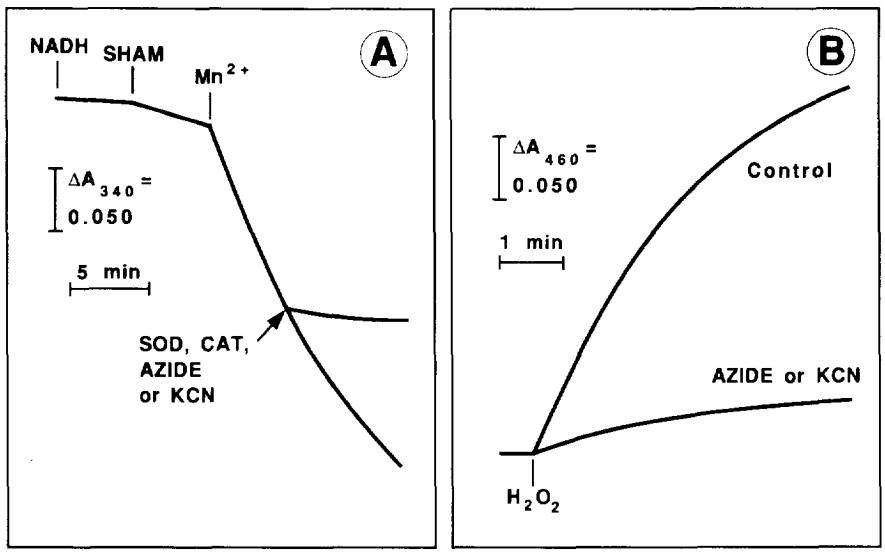

Fig. 1. Peroxidase activity in isolated soya bean plasma membranes, followed either as NADH oxidation (panel A), or as *o*-dianisidine oxidation (panel B). Additions were: 100 μM NADH; 100 μM SHAM; 1 mM $MnCl_2$; 1 mM H_2O_2; 100 IU ml^{-1} SOD or catalase (CAT); 1 mM Na-azide; 1 mM KCN.

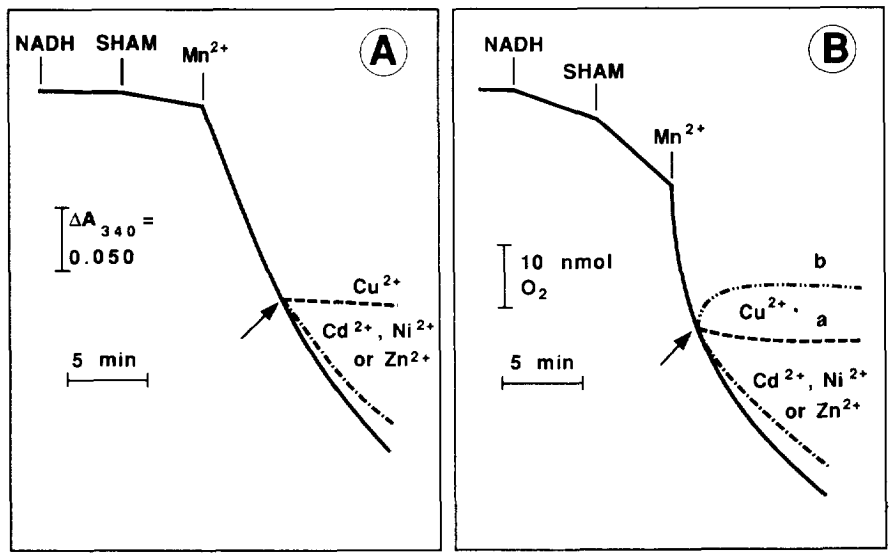

Fig. 2. Effect of divalent cations on NADH-dependent peroxidase activity of isolated soya bean plasma membranes, evaluated either as NADH oxidation or as O_2 consumption. Additions were: 100 μM NADH; 100 μM SHAM; 1 mM $MnCl_2$; 50 μM $CdCl_2$, $NiCl_2$, $ZnCl_2$; (a) 5 μM $CuCl_2$, (b) 50 μM $CuCl_2$.

membranes. These results show that the enzyme assayed was associated to soya bean plasma membranes and exhibited a peroxidase activity being able to oxidize reduced molecules in the presence of H_2O_2 or oxygen [7].

Effect of divalent cations on plasma membrane-bound peroxidase activity

Cadmium, Ni^{2+} and Zn^{2+} had little effect on SHAM plus Mn^{2+}-stimulated NADH peroxidase, evaluated either as decrease of absorbance at 340 nm (Fig. 2A) or as oxygen consumption (Fig. 2B), while 5 μM Cu^{2+} abolished it (trace a). In addition, when a higher Cu^{2+} concentration (50 μM) was used, a release of oxygen was observed (trace b). Conversely, all metals individually did not inhibit *o*-dianisidine oxidation (result not shown). This suggests that Cu^{2+} inhibited only the NADH-dependent peroxidase activity leading to H_2O_2 formation. This contention was confirmed by assaying the effect of these divalent cations on luminol-dependent chemiluminescence (H_2O_2/O_2^- generation). Figure 3 shows, indeed, that only Cu^{2+} inhibited *ca* 70% of this forma-

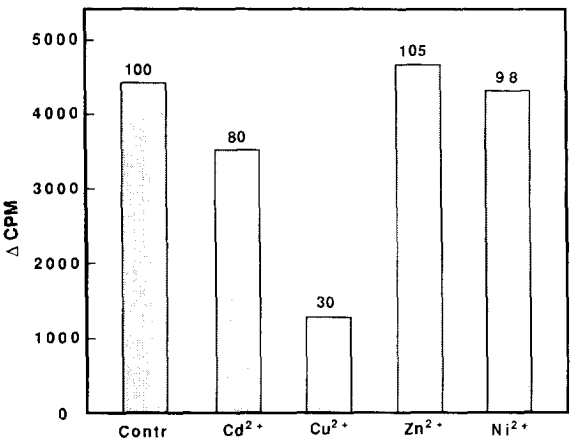

Fig. 3. Effect of divalent cations on luminol-dependent chemiluminescence (H_2O_2/O_2^- formation) in isolated soya bean plasma membranes. Figures next to each bar represent percent values. Additions were: 50 μM $CdCl_2$, $NiCl_2$, $ZnCl_2$; 5 μM $CuCl_2$.

tion, while the other cations had little (Cd^{2+}), or no effect (Zn^{2+}, Ni^{2+}).

Mechanism of Cu^{2+} inhibition

In an attempt to understand the mechanism of Cu^{2+}-induced inhibition of NADH-dependent peroxidase activity, the effect of such a cation was evaluated at increasing concentrations of NADH or Mn^{2+}. The double reciprocal plot of these results (Fig. 4) gave a K_m of 180 μM for NADH (Fig. 4A) and 240 μM for Mn^{2+} (Fig. 4B). The calculated K_i of Cu^{2+} was *ca* 600 nM and *ca* 180 nM for the two substrates, respectively. In addition, the inhibition was of a noncompetitive type in both cases, thus indicating that Cu^{2+} neither replaced Mn^{2+}, nor interacted with the catalytic site of the enzyme.

The possible involvement of sulphydryl groups in the mechanism of Cu^{2+} toxicity was also checked. Divalent copper did not influence the level of SH groups of soya bean plasma membrane and, in addition, sulphydryl-reacting compounds (*N*-ethylmaleimide, *p*-chloromercuriphenylsulphonic acid, mersalyl and 4-chloromercuribenzoic acid) had no effect on SHAM plus Mn^{2+}-stimulated NADH peroxidase activity (results not shown).

Effect of Cu^{2+} on horseradish peroxidase (HRP) activity

To confirm the above results, the effect of Cu^{2+} on commercial HRP was also assayed (Fig. 5). Figure 5(A) shows that Cu^{2+} completely inhibited SHAM plus Mn^{2+}-stimulated HRP activity and, in addition, prevented the formation of compound III (oxyperoxidase) (Fig. 5B).

DISCUSSION

Peroxidases are widely distributed in plant cells. Several workers, using different approaches, have shown that this enzyme is associated with some cell compartments. Besides vacuoles, peroxidase is mainly associated with the secretory pathway, e.g. endoplasmic reticulum, Golgi apparatus, secretory vesicles, plasma membranes and cell

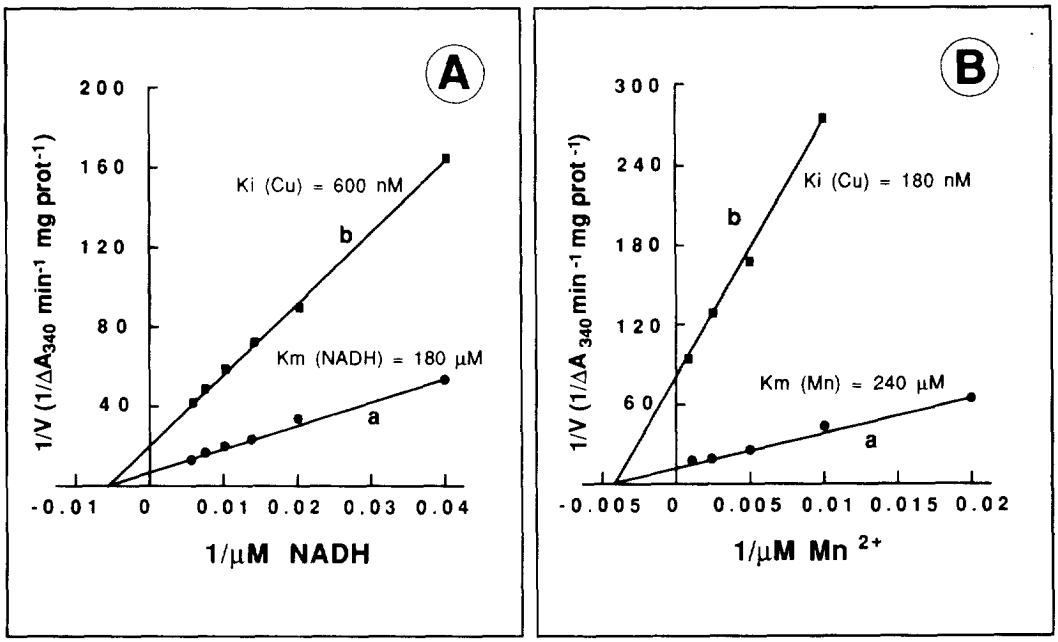

Fig. 4. Double reciprocal plot of (A) NADH-dependent peroxidase activity versus NADH, or (B) $MnCl_2$ concentration. Additions were: (a) none; (b) 1 μM $CuCl_2$.

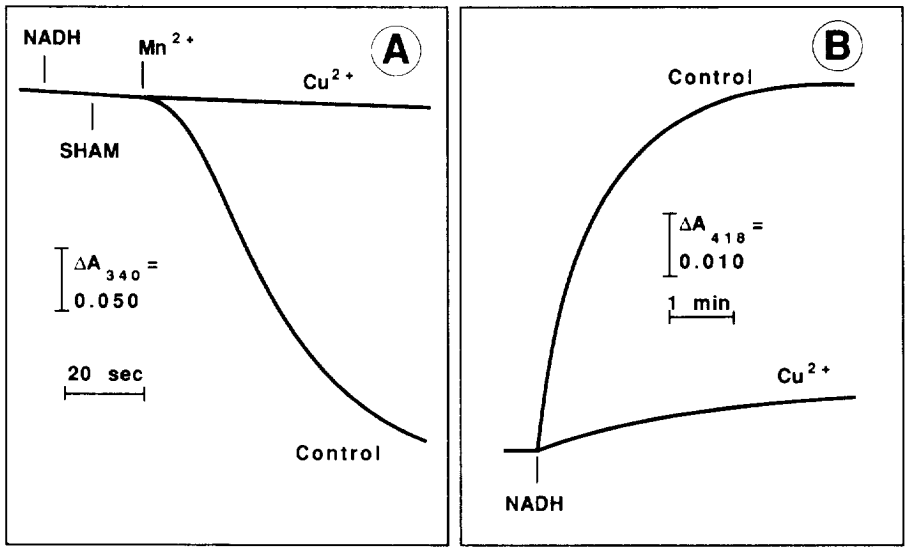

Fig. 5. Horseradish peroxidase activity, followed either as (A) NADH oxidation, or as (B) compound III formation. Additions were: (A) 100 μM NADH; (B) 2 mM NADH; 100 μM SHAM; 1 mM $MnCl_2$; 5 μM $CuCl_2$.

wall [7]. This enzyme appears to be more or less tightly bound to isolated membranes and its attachment depends on both Ca^{2+} and Mn^{2+} [15, 16]. In agreement with the results of others [14–17], those presented here show that a peroxidase, which oxidizes reduced substrates in the presence of hydrogen peroxide or oxygen, is closely associated with soya bean plasma membranes. These membranes appear to possess more than one redox system. In addition to the peroxidase here reported, an auxin-stimulated NADH oxidase [18] and a lipoxygenase [19] have been described.

Copper toxicity has been explained in a number of different ways including (i) damage of cell membranes by oxidation and cross-linking of protein thiols; (ii) inhibition of plasmalemma ATPase; and (iii) Cu^{2+}-catalysed production of free radicals which trigger lipoperoxidation [20]. Among divalent cations assayed, only Cu^{2+} strongly inhibits NADH-dependent peroxidase activity. Its effect is linked to a complete inhibition of NADH oxidation, a release of oxygen and an inhibition of H_2O_2/O_2^- formation. The mechanism by which Cu^{2+} exerts its toxicity depends neither on its reaction with sulphydryl groups, nor on a replacement of ions in the molecule.

According to Halliwell and Gutteridge [4], the reaction catalysed by a peroxidase, leading to oxidation of NADH in the presence of O_2, can be described as follows:

$$\text{NADH} + O_2 \rightarrow H_2O_2 + \text{NAD} \quad (1)$$

$$\text{Peroxidase} + H_2O_2 \rightarrow \text{Compound I} \quad (2)$$

$$\text{Compound I} + \text{NADH} \rightarrow \text{Compound II} + \text{NAD}\cdot \quad (3)$$

$$\text{Compound II} + \text{NADH} \rightarrow \text{Peroxidase} + \text{NAD}\cdot \quad (4)$$

$$\text{NAD}\cdot + O_2 \rightarrow O_2^- + \text{NAD}^+ \quad (5)$$

$$\text{Peroxidase} + O_2^- \rightarrow \text{Compound III} \quad (6)$$

$$\text{Compound III} \rightarrow \text{Peroxidase} + O_2^-/H_2O_2 \quad (7)$$

$$O_2^- + O_2^- + 2H^+ \rightarrow O_2 + H_2O_2 \quad (8)$$

$$\text{NADH} + H^+ + O_2^- \rightarrow \text{NAD}\cdot + H_2O \quad (9)$$

Copper does not seem to interact with reactions (2), (3) or (4), because these are also common to the chain of reactions leading to the breakdown of H_2O_2 [4] which, conversely, is not affected by Cu^{2+}. In addition, Cu^{2+} is not able to interact with the first step of the reaction chain. Under appropriate conditions, however, Cu^{2+} salts can both accept electrons from, and donate electrons to, the superoxide radical [4]. By this mechanism, Cu^{2+} induces the net combination of two O_2^- radicals with two H^+ ions to form H_2O_2 and O_2:

$$Cu^{2+} + O_2^- \rightarrow O_2 + Cu^+$$

$$Cu^+ + O_2^- \rightarrow O_2^{2-} + Cu^{2+}$$

$$O_2^{2-} + 2H^+ \rightarrow H_2O_2.$$

The inhibition of peroxidase activity by Cu^{2+} may, therefore, depend on an interaction with O_2^- produced in step (5):

$$O_2^- + Cu^{2+} \rightarrow Cu^+ + O_2$$

The Cu^{2+}-dependent release of O_2, observed when a higher concentration of this cation was used, can derive from this reaction. The effect of Cu^{2+} on NADH-dependent HRP activity further supports this notion. Indeed, Cu^{2+} not only inhibits this activity, but also hinders compound III formation by subtracting O_2^- to peroxidase (step 6). However, in this case, Cu^+ does not seem to react again with O_2^- and H^+ to produce H_2O_2, because this divalent cation inhibits H_2O_2 formation. It

is, however, possible that two Cu^+ may disproportionate, generating Cu^{2+} and metallic Cu:

$$Cu^+ + Cu^+ \rightarrow Cu^{2+} + \text{met Cu}$$

The inhibition of soya bean plasma membrane-bound peroxidase, caused by Cu^{2+}, can be, hence, explained by the scavenging effect exhibited by Cu^{2+} towards superoxide radicals.

EXPERIMENTAL

Plant material. Soya bean (*Glycine max* L. (Merr.), cv Visir, Pioneer) seedlings were grown for 6 days, in darkness and at 25°, 80% R.H.

Plasma membrane preparation. Hypocotyl segments (*ca* 80 g) were homogenized in 200 ml of 10 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 1 mM EDTA, 1 mM dithioerythritol, 1 mM phenylmethylsulphonyl fluoride and 0.3% (w/v) bovine serum albumin by a Ultra-Turrax apparatus, at 4°. The homogenate was filtered through 8 layers of gauze and the filtrate centrifuged at 13 000 *g* for 10 min. The supernatant was recentrifuged at 100 000 *g* for 30 min and the resulting pellet (microsomal membrane fraction) was resuspended in 5 mM NaPi buffer (pH 7.8), 0.25 M sucrose and 4 mM KCl (final volume 3 ml).

Highly purified plasma membranes were obtained from microsomes by aq. polymer two-phase partitioning, as previously described [19]. The purified plasma membranes were washed in the buffer for microsomal resuspension, centrifuged for 1 hr at 120 000 *g* and the final pellet thus obtained resuspended in the same buffer.

Peroxidase assay. Membrane-bound peroxidase or HRP were evaluated either as NADH, or as *o*-dianisidine oxidation [14]. The incubation medium was: 40 mM NaOAc (pH 5.5), 0.25 M sucrose and 200 μl of plasma membranes (*ca* 0.3 mg protein ml^{-1}) or 1 IU ml^{-1} HRP, in a final vol. of 2 ml. The reaction was started by the addition of 100 μM NADH and followed at 25° as a decrease of *A* at 340 nm. The reaction was also started by the addition of 1 mM H_2O_2 to the same buffer containing, in addition, 200 μM *o*-dianisidine, whose oxidation was followed as an increase of *A* at 460 nm.

NADH-dependent peroxidase activity was also followed as an oxygen consumption by a Clark-type oxygen electrode.

Compound III formation (oxyperoxidase) was monitored as an increase of *A* at 418 nm of medium with HRP. The reaction was started by 2 mM NADH.

H_2O_2/O_2^-. H_2O_2/O_2^- radical was detected as luminol-dependent chemiluminescence generation [21]. The incubation medium was the same used for peroxidase assay plus 10 mM luminol. The reaction was started by the addition of 100 μM NADH and proceeded at 25° for 15 min.

Sulphydryl groups. Thiol groups were determined as described in ref. [22]. The incubation medium, supplemented with 100 μM SHAM and 1 mM Mn^{2+}, was as in peroxidase assay. The reaction was started by the addition of 100 μM NADH and proceeded for 10 min at 25°. The reaction was stopped by 1% (w/v) Na dodecyl sulphate and the pH was brought to 7.6 by Tris.

Protein determination. Protein was determined by the biuret method described in ref. [23].

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