Effect of calcium and magnesium ions on radiation-induced inactivation of plant peroxidases

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Radiation-induced inactivation of soybean, horseradish, peanut, and tobacco peroxidases in the presence and in the absence of calcium and magnesium salts involves two steps which differ in the character of the effect of the metal cation. The effect of calcium cations is determined by the total charge of the enzyme molecule under experimental conditions. In general, calcium stabilizes the peroxidases, although in the case of anionic soybean and tobacco peroxidases it destabilizes the enzymes at the first inactivation step, probably due to adsorption on their surface and changes in the native conformation. The effect of magnesium ions is determined by the specific features of the enzyme structure, and the data obtained allow us to suggest the existence of additional metal-binding sites in tobacco and peanut peroxidases.

Key words: radiation-induced inactivation, horseradish peroxidase, tobacco peroxidase, soybean peroxidase, peanut peroxidase, metal ions.

The indirect effect of ionizing radiation observed in the irradiation of aqueous solutions of enzymes consists mainly in the attack of two active products of water radiolysis, *viz.*, a hydroxyl radical and a hydrated electron, on the enzyme molecule. Over the range of doses lesser than D_{80} (D_{80} is the dose at which 80% of the catalytic activity is retained), conformational changes in the active center are accompanied by the retention or loss of the catalytic activity mostly because of modification of Trp, Tyr, or His residues of the polypeptide chain. This may be considered as the first step of enzyme inactivation.

The catalytic cycle of peroxidases, which catalyze the oxidation of electron donor substrates with hydrogen peroxide, includes the consecutive formation of the so-called Compounds I and II (EI and EII) and regeneration of the original native form according to the reactions:[1]

$$E + H_2O_2 \longrightarrow EI + H_2O,$$

$$EI + S \longrightarrow EII + P,$$

$$EII + S \longrightarrow E + P + H_2O,$$

where E, EI, and EII are the native enzyme and its Compounds I and II, respectively, and S and P are the electron-donor substrate and the product of its one-electron oxidation.

In the case of radiation-induced inactivation of heme-containing peroxidases hydroxyl radicals are captured by the active center of the enzyme yielding Compound II according to the following reaction:[1]

$$E + OH^{\cdot} \longrightarrow E(OH^{\cdot}) \quad (EII).$$

Spectral studies[1] show that Compound II is present throughout the radiation-induced inactivation. However, after the irradiation is terminated the spectrum of the enzyme corresponds to that of the native form, although its catalytic activity is changed.[1]

The enzyme inactivation in the course of the peroxidase reaction has been recently shown to occur due to the interaction of Compound I with hydrogen peroxide:[2]

$$EI + H_2O_2 \longrightarrow [EI{-}H_2O_2] \longrightarrow E_i\ (P\text{-}670)$$

$$\downarrow$$

$$EII + H_2O + O_2,$$

where E_i is one of the inactivated forms of the enzyme with a characteristic visible spectrum, the so-called Compound P-670. We have not detected Compound P-670 in the course of radiation-induced inactivation[1].

Radiation-induced inactivation is characterized by accumulation of hidden damages that manifest themselves in the curves of dose-dependence of the activity as lag-periods, whose duration depends on the primary

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dose of irradiation. Dose accumulation results in irreversible stochastic destruction of amino acid residues that eventually leads to unfolding of the enzyme globule. The use of different media in the radiation-induced inactivation of peroxidases and the study of changes in the substrate specificity during radiolysis allows a detailed investigation of the catalytic mechanism of the enzyme to be performed. The effect of metal ions, in particular, calcium and magnesium, on the radiation stability and consequently on peculiarities of catalysis by tobacco peroxidase is of special interest. Earlier, we have shown by kinetic methods[3] that this peroxidase undergoes significant conformational rearrangements in the active center when its surface is saturated with calcium and magnesium ions. In addition, it is known[4] that in the case of proteinases calcium ions have a radioprotective effect. In acetate buffer, which is a weak radioprotector itself,[5] the joint effect of calcium ions and the buffer on the radiation-induced inactivation of subtilisin-72 was shown to be ionic-strength dependent.[4]

We have chosen tobacco peroxidase purified recently by us and commercially available horseradish, peanut, and soybean peroxidases for comparative studies on the effect of calcium and magnesium ions on plant peroxidases.

Results and Discussion

Inactivation of soybean (SBP), horseradish (HRP), tobacco (TOP), and peanut (PNP) peroxidases under ionizing radiation in the presence of calcium and mag-

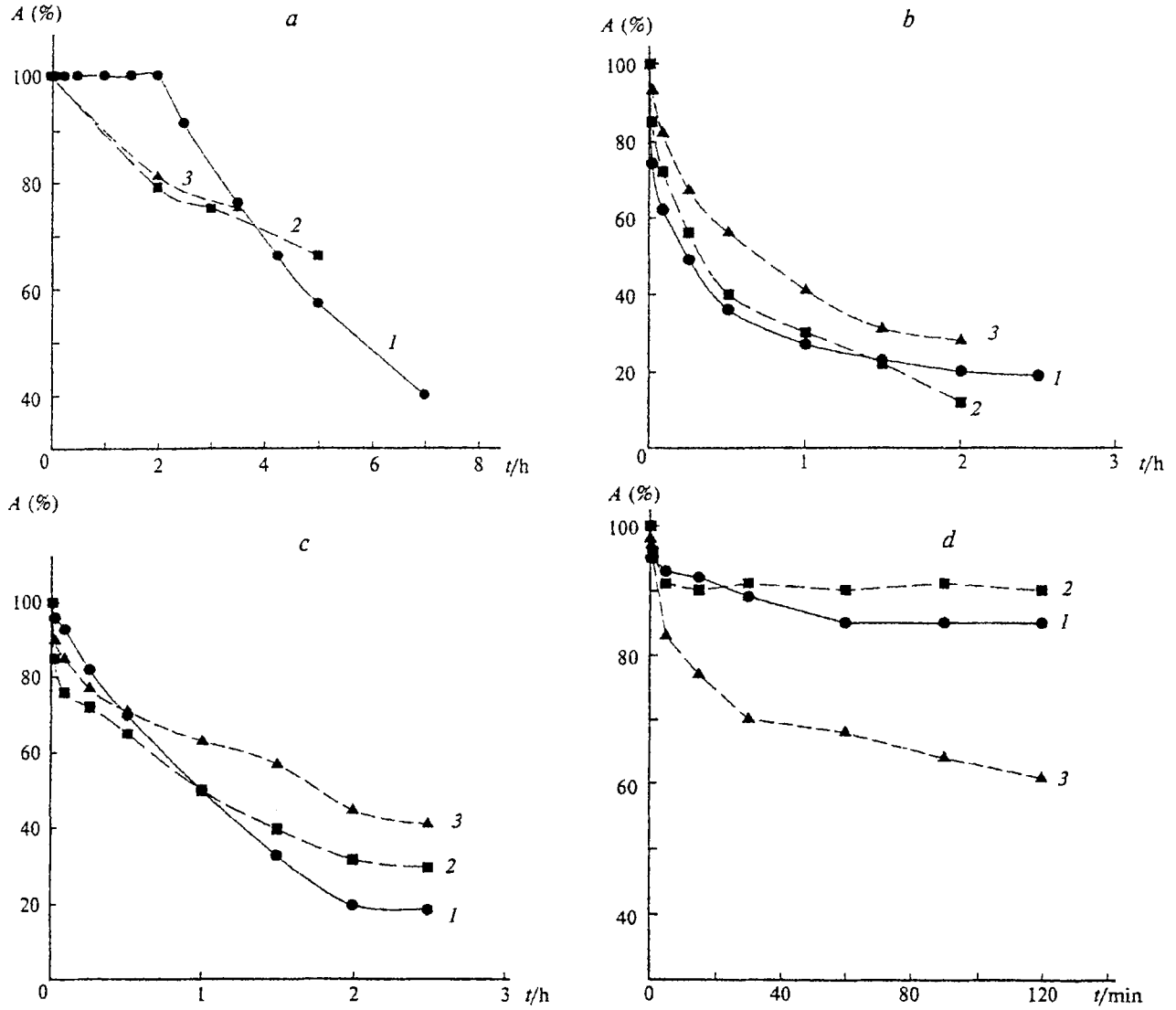

Fig. 1. Radiation-induced changes in catalytic activity (A) of SBP (*a*), HRP (*b*), TOP (*c*), and PNP (*d*) relative to the initial activity: *1*, in the absence of metal ions; *2*, in the presence of $5 \cdot 10^{-2}$ M calcium chloride; *3*, in the presence of $5 \cdot 10^{-2}$ M magnesium chloride. $P_\gamma = 180$ Gy h^{-1}.

nesium salts is presented in Figs. 1, *a*—*d*, respectively. One can see that SBP, known for its thermal stability, is also extremely stable against radiation and exhibits a lag-period up to a dose of ~360 Gy (see Fig. 1, *a*). At higher doses, the inactivation of SBP proceeds rather slowly compared to that of HRP (see Fig. 1, *b*) and TOP (see Fig. 1, *c*). The catalytic activity of SBP decreases 7—8 times more slowly than that of HRP and about 2 times more slowly than that of TOP.

Calcium and magnesium ions lead to an increase in inactivation rates in the first step of inactivation of both SBP (see Fig. 1, *a*) and TOP (see Fig. 1, *c*), although at high irradiation doses (>50—180 Gy for TOP and >600 Gy for SBP) they favor retention of the activity. The effect of metal ions on the inactivation of SBP and TOP and the character of inactivation curves (they cannot be fitted with a one-exponential dependence) point to at least two steps of radiation-induced inactivation. In the first step, saturation of the anionic enzyme surface (the total surface charge of the anionic enzyme is negative under the experimental conditions, where pH > pI, pI is the enzyme isoelectric point) with metal cations gives an additional possibility for hydrated electrons to enter the enzyme active center. Therefore, the saturation of the negatively charged surface of SBP at pH 6.0 (pI 4.1) with positive ions plays a destabilizing role. The second step is known[6] to be related to extensive modification of amino acid residues resulting in globule unfolding and scission of ionic bonds with two constitutive calcium ions found in all plant peroxidases and playing an important role in maintenance of the enzyme structure.[7] In this step, an excess of calcium or magnesium ions inhibits the inactivation process.

Calcium ions play a stabilizing role for HRP (pI 8.9), which is positively charged at pH 6.0, lowering the initial rate of inactivation ~2—3-fold; however, at doses above 50 Gy the inactivation rates in the presence and in the absence of calcium ions become comparable (see Fig. 1, *b*). Magnesium ions exhibit a more pronounced protective effect, which is not related to the difference in the ionic strength of calcium and magnesium solutions, because it was virtually unchanged during the experiments. The stabilizing effect of magnesium ions on horseradish peroxidase is first observed in this work. This seems to be due to the smaller ionic radius of magnesium cation compared to that of calcium, which favors its binding to the larger number of sorption centers in the enzyme molecule. One has to take into account the ability of calcium to form intermediate structures ("pockets") with some enzymes, for instance, with subtilisins.[8] If this holds for peroxidases, the inactivation is rather rapid at the first step because of the violation in native conformation and fixation of an altered conformation of the active center. The same reasons would enhance the stability at higher irradiation doses. This actually takes place in the case of TOP (see Fig. 1, *c*), *i.e.*, inactivation is accelerated at doses below 90—180 Gy and stabilization occurs at higher doses.

In the case of PNP, the effect of calcium ions on the enzyme stability differs from that of magnesium ions (see Fig. 1, *d*). Calcium plays a stabilizing role whereas magnesium destabilizes the enzyme.

Thus, the effect of calcium can depend mainly on the pI value of the enzyme. Calcium cations adsorbed on the surface of anionic SBP and TOP destabilize the enzyme native conformation and consequently make it more susceptible to inactivation. HRP and PNP positively charged at pH 6.0 adsorb calcium to a lesser extent and probably do not undergo the conformational changes that anionic peroxidases do. Obviously, the effect of magnesium ions characterizes the decrease in the accessible surface of both the globule and the active center (the latter fact is especially important) due to the smaller size of magnesium compared to calcium cations. In the case of PNP magnesium ions cause its dramatic destabilization, which distinguishes it from other peroxidases and probably reflects the presence of an additional metal binding site in PNP.

In the case of TOP, magnesium is a better radioprotector than calcium (see Fig. 1, *c*), although in both cases the effect is rather weak. The first inactivation step in this case is shorter than that for SBP and probably reflects the lesser conformational stability of TOP.

To evaluate the extent of conformational changes at the first inactivation step of TOP, we have determined the Michaelis constants toward hydrogen peroxide and the electron-donor substrate, ammonium 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), for enzyme preparations subjected to inactivation with different doses (15 Gy and 90 Gy) in the presence and in the absence of calcium and magnesium ions. Hydrogen peroxide is cleaved in the active center of peroxidases with the obligatory participation of the distal His-42 and Arg-38 while ABTS is oxidized *via* an electron transport chain arranged by the protein. Therefore, changes in the Michaelis constant towards hydrogen peroxide should primarily indicate modifications of the active center, while changes in the Michaelis constant toward ABTS should point to the conformational changes in the enzyme molecule affecting the heme-protein relationship.[9] The doses were selected so as to characterize both the process of enzyme destabilization by metal ions and the changes in the mechanism of their action. The values of Michaelis constants toward hydrogen peroxide and ABTS at the first inactivation step in the presence of magnesium were equal to 0.4±0.1 and 1.3±0.1 mmol L^{-1}, respectively, as could be seen in Figs. 2, *a*,*b*. In the presence of calcium ions the respective values were 0.13±0.01 and 1.28±0.05 mmol L^{-1}. Thus, the first inactivation step has almost no effect on the whole conformation of the protein globule because there are no changes in Michaelis constants toward ABTS. This is in agreement with the concept that the radiation-induced damage at the first step is associated with single-point modifications. The "lethal" modifications are localized at the active site or, as we have shown earlier,[10]

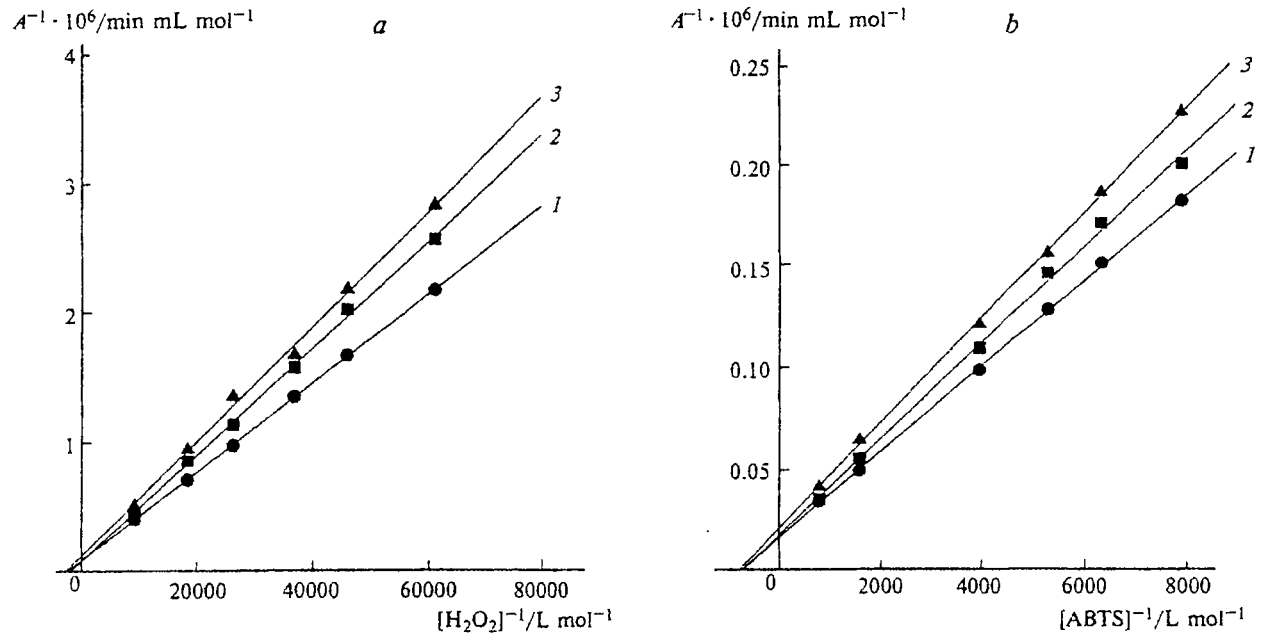

Fig. 2. Determination of the Michaelis constants towards hydrogen peroxide at a fixed concentration of ABTS equal to 0.2 mg mL^{-1} (*a*) and towards ABTS at a fixed concentration of hydrogen peroxide equal to $5 \cdot 10^{-3}$ mol L^{-1} (*b*) for TOP irradiated in the presence $5 \cdot 10^{-2}$ *M* magnesium chloride. Dose (Gy): 0 (*1*), 5 (*2*), and 45 (*3*).

affect the most radiosensitive residues, Trp and Tyr. The probability of these modifications is rather low for plant peroxidases due to their structure and amino acid sequences, *e.g.*, HRP, PNP, and TOP have 5, 6, and 3 Tyr residues, respectively, and all cloned plant peroxidases have a single Trp[7]. Thus, the enzymes are rather stable at the first inactivation step.

It is well known that the storage-inactivated enzymes are more stable than the newly purified preparations. The low specific activity seems to be the reason for the stability of PNP (*cf.* the initial specific activities of the enzymes in Table 1). Therefore, we shall discuss further only the properties of TOP and HRP, whose tertiary structures are known (in contrast to SBP) and whose specific activities are sufficiently high (>750 U mg^{-1}), indicating minimum inactivation during the storage. The differences in their stability at the first inactivation step are likely to reflect accessibility of the active center for the attack. HRP has a number of phenylalanine residues including Phe-179, -68, and -143 that can interact with hydrated electrons resulting eventually in radical modification of the active center. The active center of TOP is screened by the glutamic acid residue, Glu-141[6] (corresponding to Phe-143 in HRP) that can partially protect the active center from attack by the water radiolysis products, especially by hydrated electrons, as we discussed earlier for the HRP Phe-143→Glu mutant.[11]

As mentioned above, the Michaelis constants exhibit no changes upon radiation-induced inactivation of TOP at doses below 90 Gy; however, the Michaelis constants of the native enzyme towards hydrogen peroxide were sensitive to the presence of metal cations (*cf.* 0.4 mmol L^{-1} in the presence of magnesium and 0.13 mmol L^{-1} in the presence of calcium with 0.22±0.02 mmol L^{-1} in the absence of cations). The Michaelis constant towards ABTS is practically independent of the presence of metal cations (compare 1.3 mmol L^{-1} in the presence of cations and 1.1±0.1 mmol L^{-1} in their absence). The data obtained provide evidence for the interaction of calcium and magnesium cations with TOP resulting in conformational changes that affect the binding of hydrogen peroxide but not ABTS. In other words, the conformational changes that occur in the presence of calcium and magnesium ions affect also the active center of TOP and, hence, hydrogen peroxide binding and cleavage.

Thus, the effect of calcium on the radiation stability of peroxidase irradiation is connected both with the compensation of the negative charge on the surface of the molecule and the increase in accessibility of the active center for attack by the active particles of water radiolysis as well as with the changes in protein conformation that can cause destabilization at low doses, however, they had no effect on the ABTS-binding center in the case of TOP. The effect of magnesium on the radiation stability of peroxidase first demonstrated in this work is likely to be determined by the structure of the peroxidase active center. In the case of HRP and TOP, magnesium favors stabilization of the enzyme, in the case of SBP it has the same effect as calcium, and in the case of PNP it results in a dramatic destabilization. Presumably, a low-affinity binding site for magnesium/calcium ions adjacent to the active site exists in TOP that is in agreement with the recently made conclusions.[3]

Table 1. Characteristics of the enzyme preparations used

Peroxidase	Mw /kDa	*RZ*[a]	pI[b]	ε[c]	Specific activity[d] /U mg^{-1}
Horseradish roots	44	3.0	8.9	102000	1800
Tobacco leaves	36	3.4	3.5	108000	750
Peanut cells	40	3.2	7.8	100000	45
Soybeans	37	2.5	4.1	100000	750

[a] *RZ* is the ratio of absorbances (in o.d. units) at 403 and 280 nm characterizing the saturation of the enzyme preparation with hemin, *e.g.*, the preparation purity. [b] pI is the enzyme isoelectric point. [c] Extinction coefficient (L mol^{-1} cm^{-1}). [d] U is the unit of enzyme activity, μmol of substrate converted per minute.

Experimental

The following enzymes have been used: HRP isozyme C (Biozyme, UK), and SBP (*RZ* 1.0, Enzymol International Inc., Columbus, USA); PNP was a kind gift of Prof. R. B. van Huystee (Canada), and TOP was purified from transgenic tobacco plants according to the known protocol.[12] HRP and PNP were used without additional purification. SBP was purified by gel-filtration on a HiLoad 16/60 Superdex 75 column (Pharmacia Biotech, Sweden), equilibrated with $5 \cdot 10^{-2}$ *M* Na-phosphate buffer, pH 7.0, containing $15 \cdot 10^{-2}$ *M* NaCl. The characteristics of the homogeneous enzyme preparations used in this work are summarized in Table 1.

The activity toward ABTS was determined according to the published procedure[13] and the protein concentration was calculated using the extinction coefficients (see Table 1).

Irradiation was performed using a γ-source with dose power P_γ = 0.05 Gy s^{-1} for 10^{-7} *M* enzyme solutions in Na-acetate buffer, pH 6.0, containing $5 \cdot 10^{-2}$ *M* calcium or magnesium chloride in Eppendorf tubes. The residual enzyme activity was measured 1 h after irradiation was terminated (incubation at 22 °C).

Michaelis constants toward ABTS and hydrogen peroxide were calculated from the data on the dependence of the initial reaction rate on the concentration of the first substrate at a fixed saturating concentration of the second substrate and were plotted in double-reciprocal coordinates. Hydrogen peroxide concentration was determined spectrophotometrically, ε_{240} = 43.6 L mol^{-1} cm^{-1} (Ref. 14).

The ionic strength of solutions was measured on a Pradelkis conductometer (Hungary).

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