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Polysaccharide degradation by Fenton reaction- or peroxidase-generated hydroxyl radicals in isolated plant cell walls

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Abstract

The formation of hydroxyl radicals ($OH^{\bullet}$) by peroxidase was confirmed by EPR spectroscopy using ethanol/α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone as a spin-trapping system specific of $OH^{\bullet}$. The effect of $OH^{\bullet}$, generated either non-enzymatically with the Fenton reaction ($H_2O_2 + Fe^{2+}$) or with horseradish peroxidase in the presence of O_2 and NADH, on cell walls isolated from maize (*Zea mays*) coleoptiles or soybean (*Glycine max*) hypocotyls was investigated. $OH^{\bullet}$ produced by these reactions attack polysaccharides in the wall, demonstrated by the release of a heterogeneous mixture of polymeric breakdown products into the incubation medium. The peroxidase-catalyzed degradation of cell-wall polysaccharides can be inhibited by KCN and superoxide radical ($O_2^{\bullet-}$) or $OH^{\bullet}$ scavengers. These data support the hypothesis that $OH^{\bullet}$, produced by cell-wall peroxidases in vivo, act as wall-loosening agents in plant extension growth.

Keywords: Cell wall; Electron paramagnetic resonance (hydroxyl radical); Fenton reaction; Horseradish peroxidase; Hydroxyl radical; Peroxidase; Polysaccharide scission

1. Introduction

The production of reactive oxygen intermediates including hydroxyl radicals ($OH^{\bullet}$) in plant metabolism is generally regarded as a detrimental process causing various symptoms of oxidative stress (Halliwell and Gutteridge, 2001). Recent research into the biochemistry of oxygen radical formation indicates, however, that the controlled generation of $OH^{\bullet}$ can also serve useful physiological functions, e.g. as chemical weapons in pathogen defence (v. Tiedemann, 1997) or as cell-wall-degrading agents involved in the wall-loosening process underlying cell extension growth (Schopfer, 2001). The recent interest into this possibility has been initiated by reports demonstrating that $OH^{\bullet}$ generated by ascorbate in the presence of Cu ions can cause oxidative scission of polysaccharides such as xyloglucan or pectin (Fry, 1998) and that horseradish peroxidase (HRP) can catalyze the formation of $OH^{\bullet}$ in the presence of a suitable reductant such as NADH (Chen and Schopfer, 1999). Moreover, it has been shown that cell wall polysaccharides can be cleaved in vitro by $OH^{\bullet}$ originating from the catalytic action of HRP in the presence of O_2 and NADH (Schweikert et al., 2000). In this reaction NADH is utilized for reducing O_2 to superoxide ($O_2^{\bullet-}$) and its dismutation product H_2O_2. $O_2^{\bullet-}$ can then be used by peroxidase to reduce H_2O_2 to $OH^{\bullet}$ in a Fenton-type reaction (Chen and Schopfer, 1999). Peroxidase is normally present in the cell walls in great abundance. Moreover, there is evidence for an apoplastic production of $O_2^{\bullet-}$ and H_2O_2 in growing tissues (Frahry and Schopfer, 2001; Schopfer et al., 2001). Thus, all ingredients necessary for producing $OH^{\bullet}$ in the cell wall of such tissues are principally available under natural conditions, i.e. also in the absence of apoplastic NADH. However, the biochemical action of $OH^{\bullet}$ produced in the cell wall has so far not been examined. To fill this gap, we investigated whether $OH^{\bullet}$, experimentally generated either by the iron-catalyzed Fenton reaction or the HRP-catalyzed NADH oxidation, can cause polysaccharide degradation in the cell wall in vitro.

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2. Results and discussion

2.1. Generation of $OH^{\bullet}$ by HRP

The production of $OH^{\bullet}$ by HRP supplied with NADH in the presence of O_2 has previously been shown by EPR spectroscopy using 5,5-dimethyl-1-pyrrolidone-*N*-oxide (DMPO) as a spin trap (Schweikert et al., 2000). These experiments were complicated by the interference of $O_2^{\bullet-}$ producing an unstable DMPO–$O_2H^{\bullet}$ adduct that can be converted into the DMPO–$OH^{\bullet}$ adduct and can thus simulate the presence of $OH^{\bullet}$ (Rosen and Rauckman, 1984). This problem can be overcome by employing ethanol/α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) as a spin-trapping system that reacts with $OH^{\bullet}$, but not with $O_2^{\bullet-}$, forming a relatively stable hydroxyethyl adduct (Ramos et al., 1992). Fig. 1 shows the formation of $OH^{\bullet}$ by HRP-catalyzed NADH oxidation using ethanol/POBN as a specific spin trap for $OH^{\bullet}$. The reaction can be inhibited by inactivating peroxidase with cyanide, or by removing the intermediates $O_2^{\bullet-}$ and H_2O_2 with superoxide dismutase or catalase, respectively. These data confirm that HRP can generate $OH^{\bullet}$ in the presence of NADH and O_2 (Chen and Schopfer, 1999; Schweikert et al., 2000).

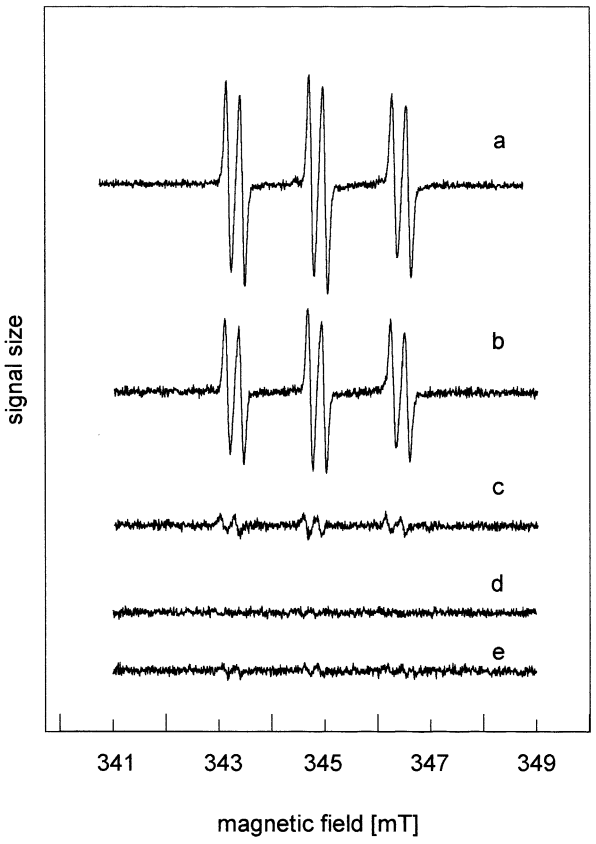

Fig. 1. Demonstration of HRP-catalyzed $OH^{\bullet}$ production by EPR spectroscopy. HRP (1.4 μM) and NADH (0.2 mM) were incubated for 5 min in phosphate buffer in the presence of ethanol/POBN. The EPR spectrum of the hydroxyethyl-POBN adduct formed (b) matches the spectrum obtained from $OH^{\bullet}$ produced by the Fenton reaction ($H_2O_2 + FeSO_4$) in the presence of ethanol/POBN (a). $OH^{\bullet}$ production by HRP in the presence of NADH can be inhibited by KCN (10 mM) (c), as well as by eliminating either $O_2^{\bullet-}$ or H_2O_2 with superoxide dismutase (100 μg ml^{-1}) (d) or catalase (100 μg ml^{-1}) (e), respectively.

2.2. Cell-wall degradation with $OH^{\bullet}$ generated by the Fenton reaction

Two types of primary cell wall with greatly differing matrix polysaccharides can be distinguished in flowering plants (Carpita and Gibeaut, 1993): Type I primary walls, found in dicots and non-graminean monocots, contain xyloglucan and pectin as major matrix polymers. In contrast, the matrix of type II primary walls of grasses (Poaceae) is primarily composed of glucuronoarabinoxylans and (β-1,3),(β-1,4)-glucans. Despite of these qualitative chemical differences both types of wall respond very similarly to wall loosening-inducing cues, such as the phytohormone auxin, causing cell-wall expansion in growing tissues. This suggests that chemical wall loosening can be brought about by a reaction mechanism that indiscriminately causes the breakage of intra- or inter-molecular bonds in all existing kinds of matrix polymers of primary walls. A suitable candidate for a wall-loosening factor capable of unspecific scission of polysaccharides is the promiscuously reactive $OH^{\bullet}$ (Fry, 1998; Schweikert et al., 2000). For investigating the potential of $OH^{\bullet}$ for degrading cell walls of growing plant organs in vitro, we used the coleoptile of maize seedlings (type II wall) and the hypocotyl of soybean (type I wall) as representative sources for preparing cell wall material as a target substrate for the action of $OH^{\bullet}$.

Fig. 2 shows that $OH^{\bullet}$ produced from H_2O_2 in the presence of iron ions (Fenton reaction) mediates the solubilization of polysaccharides in both types of wall. These data demonstrate that $OH^{\bullet}$ is principally capable of degrading polysaccharides, still integrated in the cell-wall structure, to such an extent that soluble fragments are released into the incubation medium where further cleavage may take place. The size distribution of polysaccharide fragments obtained after 20 h of incubation is shown in Fig. 3. These data show that the attack by $OH^{\bullet}$ in the cell wall results in the release of a complex mixture of cleavage products in the range of 1 to $\geqslant$1000 kDa molecular mass. Moreover, the chromatographic profiles reveal characteristic differences between maize and soybean walls with respect to the size distribution of cleavage products. Under the particular conditions of these experiments, maize walls release a major fraction of $\geqslant$1000 kDa followed by successively decreasing amounts of fractions of lower molecular mass. Treated in the same way, soybean walls produce a narrow peak at $\geqslant$1000 kDa and a broad, heterogeneous peak between 1 and 10 kDa.

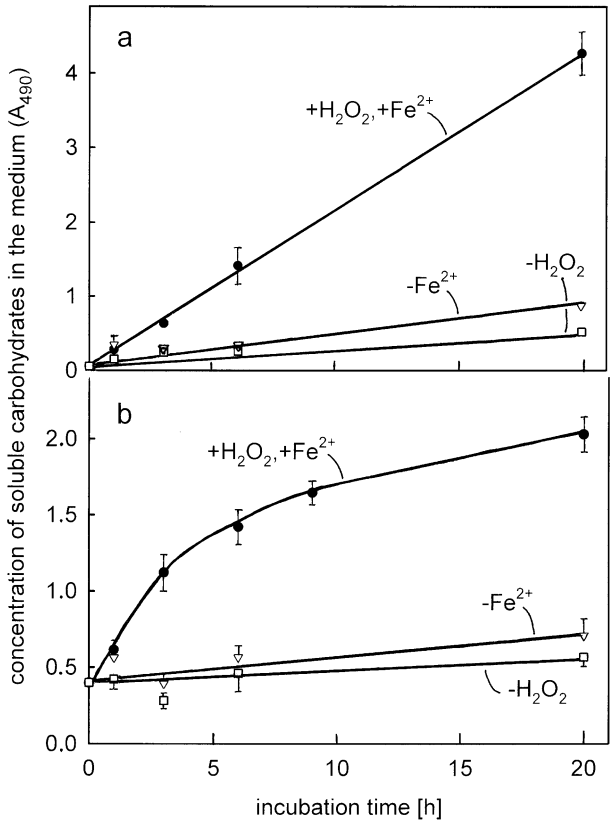

Fig. 2. Release of soluble carbohydrates from cell walls by OH• generated in a Fenton reaction. Purified, heat-inactivated wall preparations (10 mg in 1 ml) from maize coleoptiles (a) and soybean hypocotyls (b) were incubated with 100 mM H_2O_2 and 100 μM $FeSO_4$ in citrate buffer (pH 6.0) for up to 20 h. Controls without H_2O_2 ($-H_2O_2$) or $FeSO_4$ ($-Fe^{2+}$) were run in parallel. $A_{490} = 1$ is equivalent to a sucrose concentration of 1.3 mM in the incubation medium.

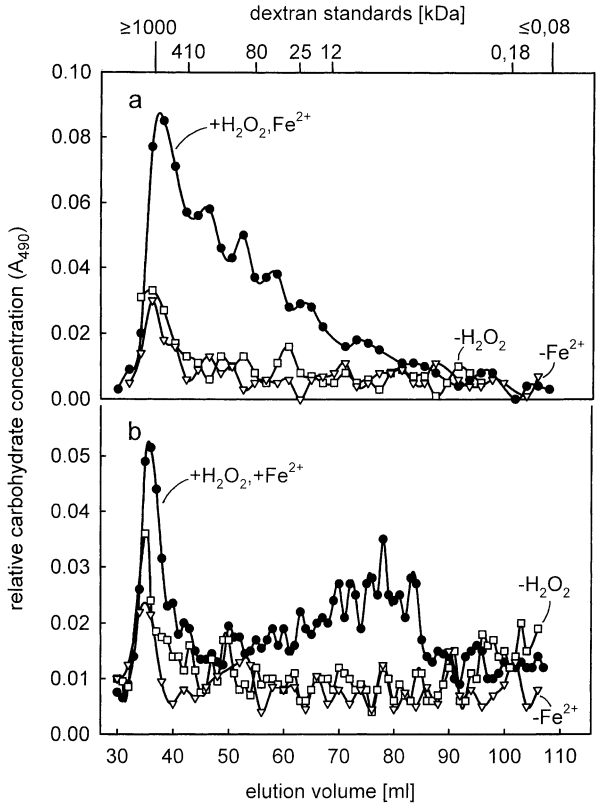

Fig. 3. Elution profiles obtained after size fractionation of carbohydrates released from 5 mg cell wall of maize coleoptiles (a) or soybean hypocotyls (b) incubated in a OH•-generating Fenton reaction system for 20 h as in Fig. 2. Controls without H_2O_2 ($-H_2O_2$) or $FeSO_4$ ($-Fe^{2+}$) were run in parallel. The elution pattern of molecular-mass standards (dextran) is indicated at the top.

2.3. Cell-wall degradation with OH• generated by HRP

In the cell walls of the living plant OH• can be produced by peroxidase in the presence of $O_2^{\bullet-}$ and H_2O_2, presumably provided by a plasma membrane NADH oxidase (Schopfer et al., 2002). In vitro, this peroxidase function can be stimulated by utilizing the ability of the enzyme to act as a $O_2^{\bullet-}/H_2O_2$-producing NADH oxidase itself (Yokota and Yamazaki, 1965). We used this pharmacological reaction for investigating the question whether OH•-dependent cell-wall degradation in vitro can be achieved by the catalytic action of peroxidase. Fig. 4 shows the size distribution of the cleavage products released from maize or soybean cell walls incubated with HRP and NADH. It is apparent from these data that the generation of OH• by peroxidase causes the cleavage and release of cell-wall polysaccharides similarly to the Fenton reaction in both types of cell wall, although the size distribution of products is somewhat different.

Additional information on HRP-catalyzed cell-wall degradation is depicted in Fig. 5. The addition of H_2O_2 to the reaction mixture increases the release of high-molecular-mass products from the wall and reduces the appearance of smaller fragments (Fig. 5a). The OH• scavengers histidine and benzoate (Halliwell and Gutteridge, 2001; Schweikert et al., 2000) suppress the release of soluble polysaccharides from the wall (Fig. 5b). Moreover, the reaction can be inhibited by inactivating the peroxidase with KCN, or by removing $O_2^{\bullet-}$, an intermediate in the production of H_2O_2 and OH• by peroxidase in the presence of NADH (Chen and Schopfer, 1999), with Mn-desferrioxamine, a chemically stable substitute for superoxide dismutase (Rabinowitch et al., 1987; Able et al., 1998). These results confirm that OH• is involved in the peroxidase-catalyzed degradation of cell-wall polysaccharides and that $O_2^{\bullet-}$ and H_2O_2 are essential intermediates in this reaction.

Taken together, our results show that cell-wall degradation by chemical polysaccharide degradation can be mediated by OH• and that the production of OH• in the cell wall can be catalyzed by peroxidase in vitro. Polysaccharide degradation by OH• produces a mixture of polymeric fragments, in contrast to the autolytic degradation of wall polysaccharides by exo- and endo-glucanases which results primarily in a complete breakdown to monosaccharides (Huber and Nevins, 1980; Hohl et al., 1991). This difference may be a useful criterion for

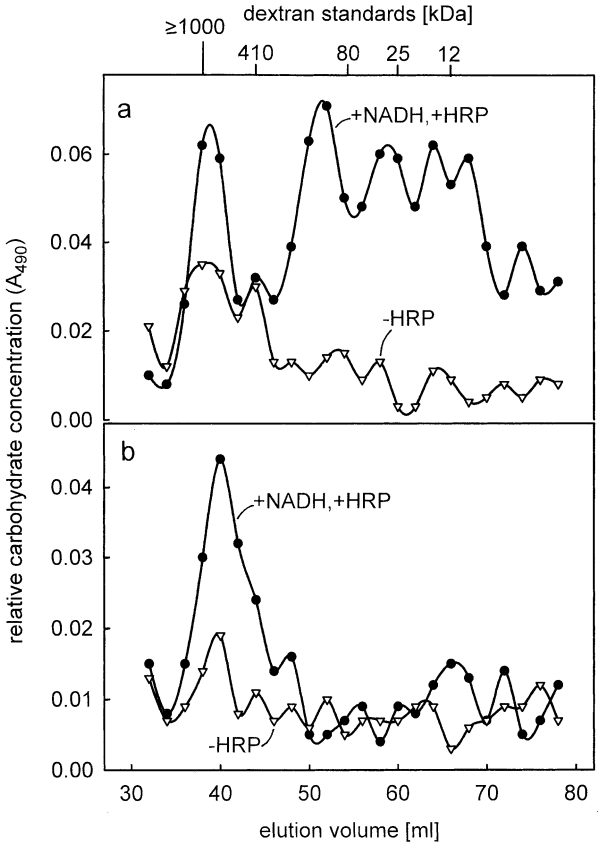

Fig. 4. Elution profiles obtained after size fractionation of carbohydrates released from cell walls by OH• generated by HRP in the presence of NADH. Cell wall material (20 mg) from maize coleoptiles (a) or soybean hypocotyls (b) was incubated with 2.0 or 0.5 mM NADH (maize or soybean walls, respectively) and 1.4 μM HRP in citrate buffer (pH 6.0) for 20 h. Controls without HRP (–HRP) were run in parallel.

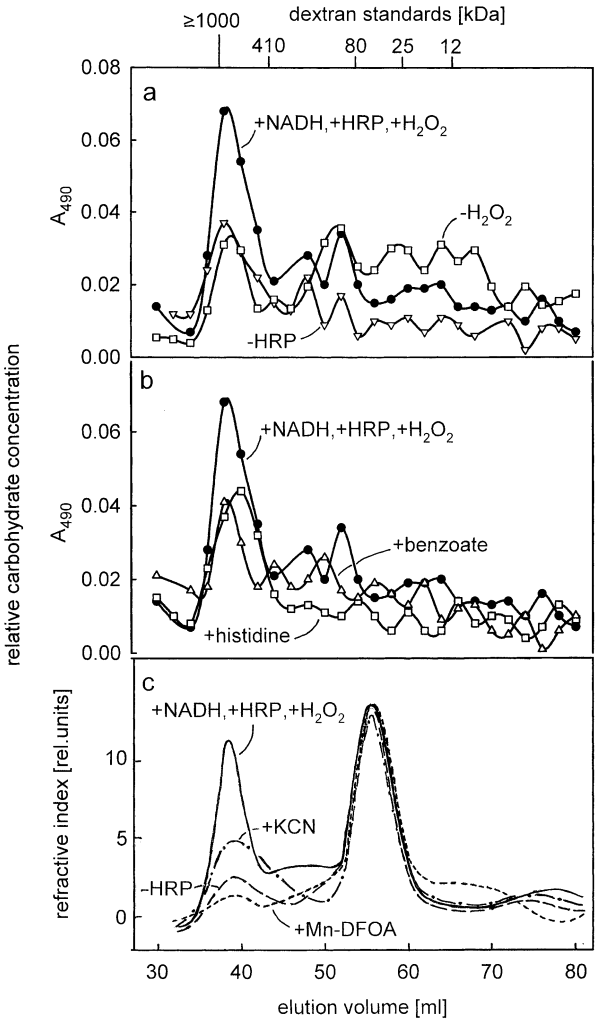

Fig. 5. Promotion by H_2O_2 (a) and inhibition by histidine or benzoate (OH• scavengers) (b), KCN (peroxidase inhibitor) (c), and Mn-desferrioxamine (Mn–DFOA, $O_2^{\bullet-}$ scavenger) (c) of HRP/NADH-mediated carbohydrate degradation in maize cell walls. Cell-wall material (10 mg) was incubated with NADH and HRP as in Fig. 4a with the following additions: 10 mM H_2O_2 (a), 10 mM H_2O_2 + 100 mM histidine or 100 mM benzoate (b), 10 mM H_2O_2 + 10 mM KCN or 0.4 mM Mn–DFOA (c). Elution profiles were determined as in Fig. 4 except that the refractive-index profile instead of the A_{490} profile is shown in panel c because Mn-DFOA interferes with the A_{490} measurement. The refractive-index profiles show an additional peak due to catalase included for the destruction of residual H_2O_2 at the end of the reaction. One relative unit is equivalent to a sucrose concentration of 6.23 μM.

discriminating between enzymatic and non-enzymatic, OH•-mediated wall degradation.

3. Experimental

3.1. Materials and special chemicals

Purified cell-wall material was prepared by homogenizing coleoptiles or hypocotyls, respectively, of 5-d-old dark-grown seedlings of maize (*Zea mays* L., cv. Perceval) or soybean (*Glycine max* L., cv. Recor R1 NT) seedlings in liquid nitrogen using a ball mill (Müsel et al., 1997). The cell-wall residue was washed twice with homogenization buffer, twice with 80% acetone, twice with pure acetone and lyophilized. Peroxidase (EC 1.11.1.7, horseradish, type VI), POBN and dextran standards for calibrating the chromatography column were from Sigma-Aldrich-Fluka (Deisenhofen, Germany), superoxide dismutase (EC 1.15.1.1, bovine erythrocytes) and catalase (EC 1.11.1.6, beef liver) from Roche Molecular Biochemicals (Mannheim, Germany). Mn-desferrioxamine was prepared from desferrioxamine mesylate (Sigma) and MnO_2 according to Beyer and Fridovich (1989).

3.2. Assay of polysaccharide release from the cell wall

Samples of 10–30 mg purified cell wall were suspended in 1–3 ml Na–citrate buffer (10 mM, pH 6.0) and boiled for 20 min for inactivating wall enzymes. Solubilized material was removed by washing three

times with buffer. The remaining cell-wall residue was suspended in buffer (0.1 ml per mg cell wall) and incubated with various additions at 30 °C on a shaker. At suitable time intervals aliquots were removed and the concentration of total sugars was determined photometrically (A_{490}) by the phenol-sulfuric acid method (Dubois et al., 1956) in 0.1-ml-samples of supernatant obtained after centrifugation. As H_2O_2 interferes with this assay, it was removed by incubating the supernatant with 0.2 mg ml^{-1} catalase for 15 min before adding the Dubois reagents. Data points represent means of at least four independent experiments ±estimated SE.

3.3. Size exclusion chromatography

Solubilisized cell wall material was fractionated on a Fractogel EMD Biosec column (59×2 cm, separation range 10^2–10^6 Da; Merck, Darmstadt, Germany), eluted (1 ml min^{-1}) with 20 mM Na-phosphate buffer (pH 7.2) containing 0.3 M NaCl using an FPLC-System (Pharmacia, Uppsala, Sweden). The carbohydrate concentration of the eluate was monitored with a refractory index detector (ERC-7515A, ERC, Altegolfsheim, Germany) as well as in collected fractions with the Dubois assay. Both methods produced very similar elution profiles. Typical results of at least three independent runs are shown.

3.4. EPR spectroscopy

The production of $OH^{\bullet}$ by HRP was analyzed by incubating the enzyme for 15 min with 0.2 mM NADH in K-phosphate buffer (20 mM, pH 6.0) containing 170 mM ethanol/10 mM POBN as spin-trapping reagent (Ramos et al., 1992). EPR spectra of the hydroxyethyl-POBN adduct formed in the incubation medium were recorded at room temperature in a flat cell at 63 mW microwave power, 100 kHz modulation frequency, 0.2 mT modulation amplitude, 9.687 GHz microwave frequency in a Bruker ESP X-band spectrometer. For the Fenton reaction 10 μM Fe^{2+}-EDTA was mixed with 1 mM H_2O_2 in buffer and incubated for 5 min together with ethanol/POBN.

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