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Scission of polysaccharides by peroxidase-generated hydroxyl radicals

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Abstract

Cell-wall polysaccharides can be broken down non-enzymatically in vitro by scission of backbone bonds in a Fenton reaction system producing hydroxyl radicals ($OH^{\cdot}$) (Fry, S.C. (1998). *Biochemical Journal*, 332, 507–515). $OH^{\cdot}$ can also be generated enzymatically from O_2 by horseradish peroxidase (HRP) in a complex reaction cycle involving NADH or dihydroxyfumarate (DHF) as reducing substrate (Chen, S.-X., & Schopfer, P. (1999). *European Journal of Biochemistry*, 260, 726–735). Based on these recent findings the possibility that HRP can be used to degrade cell-wall polysaccharides in vitro was investigated. The production of $OH^{\cdot}$ from O_2 by HRP in the presence of NADH or DHF was confirmed by EPR spectroscopy using 5,5-dimethyl-1-pyrroline-*N*-oxide as a spin trap. Chemical scission of polysaccharides (dextran, pectin, xyloglucan) by HRP-generated $OH^{\cdot}$ was demonstrated using a viscometric assay. The reaction could be inhibited by an array of $OH^{\cdot}$ scavengers, confirming the involvement $OH^{\cdot}$ as the causative agent for macromolecule cleavage. The significance of these findings for the biochemical function of peroxidase in cell-wall loosening processes underlying cell expansion and related physiological processes is discussed.

Keywords: Electron paramagnetic resonance (hydroxyl radical); Horseradish peroxidase; Hydroxyl radical; Pectin; Peroxidase; Polysaccharide scission; Xyloglucan

1. Introduction

Hydroxyl radicals ($OH^{\cdot}$) are short-lived, extremely reactive molecules capable of degrading all kinds of organic molecules in a diffusion-limited reaction (Halliwell & Gutteridge, 1989; Hippeli & Elstner, 1997). Production of $OH^{\cdot}$ by Fenton's reagent (H_2O_2 + catalytic amounts of $FeSO_4$) can be used to decompose cotton fibres and other cellulose-containing materials such as straw and sawdust (Halliwell, 1965). There is indirect evidence that $OH^{\cdot}$ plays a decisive role in cell damage and death caused under conditions of oxidative stress, although the generation of $OH^{\cdot}$ in vivo has only rarely been demonstrated in plants (Kuchitsu, Kosaka, Shiga & Shibuya, 1995; v.Tiedemann, 1997). Extending preliminary experiments by Miller (1986) and Fry (1998) showed that cell-wall polysaccharides such as pectin and xyloglucan can be broken down in vitro by $OH^{\cdot}$ generated in a non-enzymatic Fenton-type reaction, e.g. by the reduction of O_2 with ascorbate in the presence of Cu ions. This finding led Fry (1998) to suggest that $OH^{\cdot}$, produced in the apoplastic space of plant tissues, could act as a site-specific oxidant targeted to play a useful physiological role in cell-wall loosening processes underlying cell expansion, fruit ripening and organ abscission. Independently, similar ideas have been put forward based on the finding that plant peroxidases, e.g. horseradish peroxidase (HRP) can catalyze the production of $OH^{\cdot}$ from O_2 in the presence of a suitable reductant such as NADH (Chen & Schopfer, 1999). Under these particular conditions the enzyme initiates a complex reaction cycle

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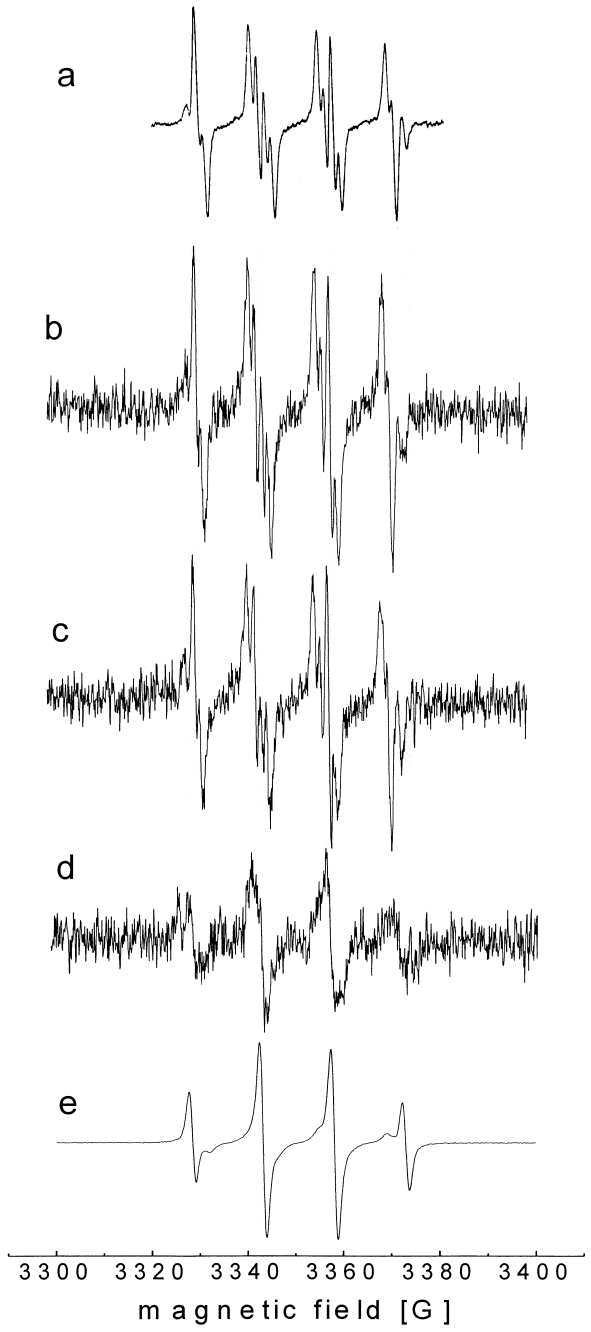

Fig. 1. Demonstration of radical generation by HRP in the presence of NADH or DHF by EPR spectroscopy at room temperature. HRP (0.7 μM) was mixed with 200 μM NADH (b), 200 μM DHF (c), or 200 μM DHF followed after approximately 15 s by 100 μg · ml^{-1} superoxide dismutase (d) in 10 mM citrate buffer (pH 5.5). Then 50 mM DMPO was added and the EPR spectra of the DMPO radical adducts recorded after approximately 2 min. Spectra of the DMPO-OH· adduct (e) produced by the Fenton reaction (1mM $FeSO_4$ + 10 mM H_2O_2) and the DMPO-O_2H· adduct (a; adopted from Rosen & Rauckman, 1984) are shown for comparison

involving superoxide radical ($O_2^{\cdot-}$) and H_2O_2 as intermediates:

$$NADH + 1/2\ H_2O_2 \rightarrow NAD^{\cdot} + H_2O \quad (1)$$

$$NAD^{\cdot} + O_2 \rightarrow NAD^{+} + O_2^{\cdot-} \quad (2)$$

$$NADH + H^{+} + O_2^{\cdot-} \rightarrow NAD^{\cdot} + H_2O_2 \quad (3)$$

$$O_2^{\cdot-} + H_2O_2 \rightarrow OH^{\cdot} + OH^{-} + O_2 \quad (4)$$

Peroxidases are abundantly present in plant cell walls where they are partly bound to polysaccharides (Everse, Everse & Grisham, 1990/1991). This offers the possibility for a site-specific generation of OH· at the right place for the cleavage of load-bearing polysaccharides and thus for a controlled weakening of the cell-wall structure without damaging other cell constituents.

Taken together, these ideas provide a novel, experimentally testable working hypothesis for the biochemical mechanism of cell growth caused by wall loosening. In a first attempt to examine this hypothesis, we investigated whether peroxidase-mediated OH· production can be utilized for cleaving polysaccharide chains in vitro.

2. Results and discussion

We have previously shown that HRP can catalyze the reduction of O_2 to OH· in a complex reaction cycle involving NADH or dihydroxyfumarate (DHF) as a reducing substrate and H_2O_2 and $O_2^{\cdot-}$ as intermediates (Chen & Schopfer, 1999). Production of OH· by this enzymatic system was demonstrated by two indirect assay procedures, based on the hydroxylation of benzoate and the degradation of deoxyribose (Gutteridge, 1987). Fig. 1 confirms this result by providing EPR spectra of the radical species formed in the reaction of HRP with O_2 and NADH or DHF, respectively, using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as a spin trap for short-lived oxygen radicals. OH· and $O_2^{\cdot-}$ react covalently with DMPO giving rise to much more stable secondary adduct radicals that can be detected at room temperature using conventional EPR spectroscopy (Rosen & Rauckman, 1984; Knecht & Mason, 1993). The DMPO-OH· adduct produces a 4-line spectrum with a 1:2:2:1 intensity ratio (Fig. 1e) whereas the DMPO-O_2H· adduct produces a more complex set of 12 lines (Fig. 1a) (Rosen & Rauckman, 1984). HRP incubated with NADH or DHF in the presence of O_2 at pH 5.5 produces similar EPR spectra in which the lines characteristic for the DMPO-OH· adduct are superimposed by the DMPO-O_2H· spec-

trum and therefore not clearly discernible (Fig. 1b, c). This is to be expected as $O_2^{\cdot-}$ is formed as an intermediate in the OH·-producing reaction cycle of HRP (Chen & Schopfer, 1999). Superoxide dismutase added to the ongoing reaction largely suppresses the appearance of the $O_2^{\cdot-}$ adduct and allows the OH· adduct to become visible (Fig. 1d). However, at the same time the interruption of $O_2^{\cdot-}$ accumulation impairs the production of OH· which depends on traces of $O_2^{\cdot-}$ escaping dismutation. Thus, the experiment illustrated in Fig. 1d greatly underestimates the OH· generation by HRP in the absence of superoxide dismutase. Similar results were obtained at pH 5.0 and 6.0, although signal intensity was reduced by 20% and 40%, respectively. The identification of OH· with DMPO in the presence of large amounts of $O_2^{\cdot-}$ can be complicated by the slow spontaneous conversion of DMPO-O_2H· into DMPO-OH· which produces a background level of DMPO-OH· of approximately 3% relative to DMPO-O_2H· (Rosen & Rauckman, 1984). This effect should be negligible with the short incubation time used in the experiments of Fig. 1. In addition, we performed similar experiments with the phosphorylated DMPO analog 5-(diethoxyphosphoryl)-5-methyl-1-pyrrolidone-*N*-oxide (DEPMPO) that also detects both oxygen radical species but is not liable to artifactual

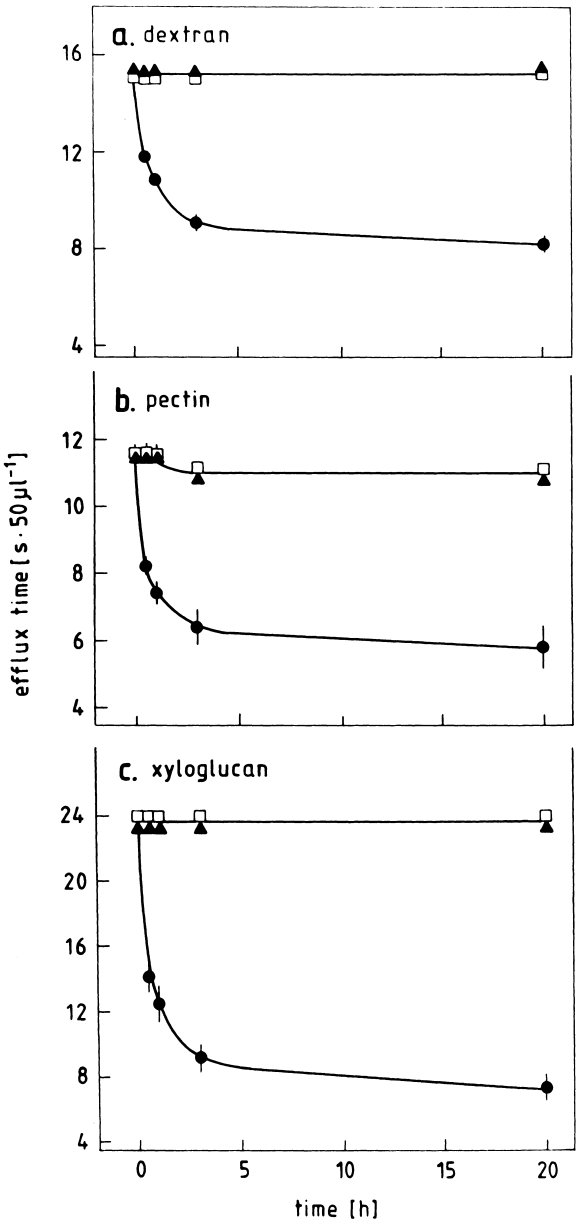

Fig. 2. Polysaccharide scission by HRP in the presence of NADH. Solutions containing 16 g · l^{-1} dextran (a), 8 g · l^{-1} pectin (b), or 7 g · l^{-1} xyloglucan (c) in citrate buffer (pH 6.0) were incubated with 4 mM NADH (□), 1.4 µM HRP (▲) or 4 mM NADH + 1.4 µM HRP (●) and the changes in viscosity (viscometer efflux time) determined after various time intervals

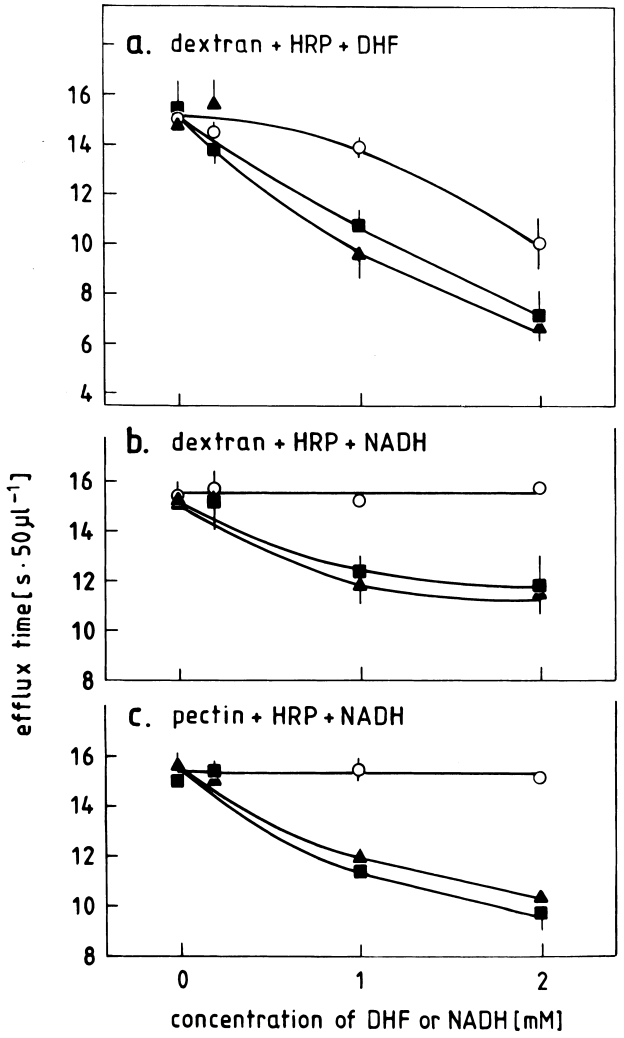

Fig. 3. Effect of DHF and NADH on peroxidase-mediated polysaccharide scission. Solutions containing 16 g · l^{-1} dextran (a, b) or 8 g · l^{-1} pectin (c) were incubated at pH 6.0 with 0 µM (○), 1.4 µM (■) or 2.8 µM (▲) HRP at various concentrations of DHF (a) or NADH (b, c) and the changes in viscosity determined after 1 h

production of the OH· adduct from the $O_2^{\cdot-}$ adduct (Roubaud, Sankarapandi, Kuppusamy, Tordo & Zweier, 1997). Experiments with DEPMPO performed similarly as shown in Fig. 1 provided qualitatively similar results although OH· accumulation was less than observed with DMPO (data not shown).

Chemical scission of polysaccharide chains can conveniently be determined by monitoring the decrease in viscosity of appropriate solutions (Freifelder, 1982). This method was adopted for testing the effect of peroxidase-generated OH· on the integrity of high mol. wt. dextran and the cell-wall polysaccharides pectin and xyloglucan. Fig. 2 shows that all three polymers can be cleaved into fragments of lower mol. wt. by HRP + NADH under aerobic conditions. Similar results were obtained using DHF instead of NADH as reducing substrate, although, at higher concentration, DHF was slightly active also in the absence of HRP (Fig. 3a). This effect is presumably due to the high susceptibility of DHF to autoxidation, leading to the spontaneous production of H_2O_2 that can be further reduced to OH· in the presence of traces of transition metals as in the case of ascorbate (Fry, 1998). Under the experimental conditions used, HRP-mediated poysaccharide scission was saturated at 1.4 μM HRP (Fig. 3). The reaction was little affected by H^+ concentration in the range of pH 4–6, except that pH 4 slightly promoted scission by autoxidation of NADH in the absence of HRP and inhibited the action of HRP on pectin, but not on dextran (Table 1).

A critical test for the involvement of OH· in polymer scission is the inhibition of this reaction by OH· scavengers effectively competing with the macromolecular targets for OH· (Halliwell & Gutteridge, 1989). As an example, Fig. 4 shows that benzoate, a very effective

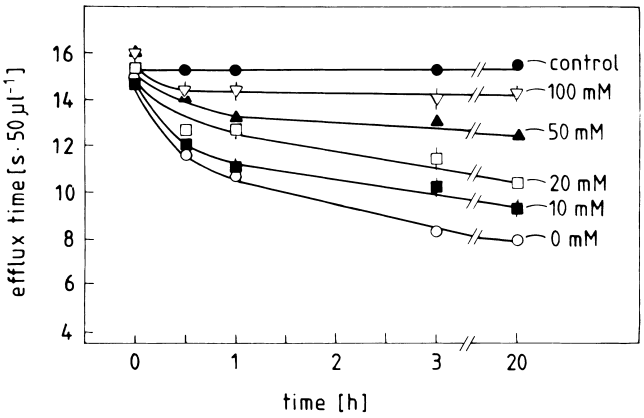

Fig. 4. Effect of the OH· scavenger benzoate on HRP/NADH-mediated polysaccharide scission. Solutions containing 16 g · l^{-1} dextran, 1.4 μM HRP and 4 mM NADH were incubated at pH 6.0 with Na-benzoate concentrations as indicated and the changes in viscosity determined after various time intervals. The control contained only dextran + NADH

and specific OH· scavenger, protects dextran from HRP-mediated scission in a concentration-dependent manner in the range of 10–100 mM. Data demonstrating the effectiveness of a selection of other established OH· scavengers (Halliwell & Gutteridge, 1989) are compiled in Table 2. Urea, known to possess no appreciable scavenger activity in contrast to thiourea (Halliwell & Gutteridge, 1989) was included as a nega-

Table 1
Effect of pH on HRP-mediated polysaccharide scission. Solutions containing 16 g · l^{-1} dextran or 8 g · l^{-1} pectin were incubated in citrate buffer for 1 h in the presence of 2 mM NADH with or without 1.4 μM HRP

		Efflux time (s · 50 μl^{-1})	
		0 h	1 h
pH 4.0	Dextran, − HRP	16.3 ± 0.7	13.8 ± 4.0
	Dextran, + HRP	–	9.0 ± 1.3
	Pectin, − HRP	15.3 ± 0.4	12.5 ± 2.2
	Pectin, + HRP	–	13.0 ± 1.0
pH 5.0	Dextran, − HRP	15.2 ± 1.1	14.4 ± 1.1
	Dextran, + HRP	–	11.5 ± 1.4
	Pectin, − HRP	15.3 ± 1.0	16.4 ± 1.5
	Pectin, + HRP	–	10.0 ± 1.5
pH 6.0	Dextran, − HRP	15.3 ± 0.5	13.7 ± 2.9
	Dextran, + HRP	–	9.2 ± 1.0
	Pectin, − HRP	15.3 ± 0.2	15.3 ± 0.0
	Pectin, + HRP	–	9.8 ± 0.7

Table 2
Effect of OH· scavengers on HRP-mediated polysaccharide scission. Solutions containing 16 g · l^{-1} dextran were incubated at pH 6.0 for 20 h in the presence of 4 mM NADH, 1.4 μM HRP and scavengers as indicated. Urea, having very little OH·-scavenging activity in contrast to thiourea, was included as a negative control

	Efflux time (s · 50 μl^{-1})	Inhibition (%)
Controls		
a. − NADH, − HRP	15.3 ± 0.1	100
b. + NADH, + HRP	8.6 ± 0.2	0
Reagents added to b:		
Na-benzoate, 100 mM	13.9 ± 0.2	79
Na-formate, 100 mM	11.3 ± 0.3	40
Na-formate, 200 mM	12.4 ± 0.5	56
Na-formate, 500 mM	15.0 ± 0.2	96
Na-lactate, 100 mM	13.6 ± 0.3	74
Tris, 100 mM	14.5 ± 0.3	88
Glucose, 500 mM	14.3 ± 0.4	85
Mannitol, 250 mM	12.3 ± 0.5	55
Mannitol, 500 mM	13.9 ± 0.3	79
Mannitol, 1000 mM	15.0 ± 0.2	95
DMSO, 100 mM	11.9 ± 0.2	49
DMSO, 500 mM	13.1 ± 0.0	67
Histidine, 10 mM	9.3 ± 0.0	10
Histidine, 20 mM	11.0 ± 0.1	35
Histidine, 200 mM	15.2 ± 0.1	98
Thiourea, 100 mM	15.1 ± 0.1	97
Urea, 100 mM	9.1 ± 0.5	7

tive control. These data demonstrate that the HRP-mediated scission of polysaccharide chains depends on the formation of $OH^{\cdot}$ that is obviously the causative agent for macromolecule cleavage.

In conclusion the present report provides evidence that the generation of $OH^{\cdot}$ by peroxidase in the presence of O_2 and a suitable reductant in vitro is sufficient for degrading cell-wall polysaccharides by cleavage of backbone bonds. This finding is of significance with regard to the ill-defined function of peroxidase in vivo. Peroxidase is a secretory enzyme generally present in a high concentration in the cell walls of growing or maturing plant tissues (Everse et al., 1990/1991) where it is thought to be involved in H_2O_2-mediated cross-linking of phenolic wall components (Fry, 1986; Wojtaszek, 1997) and wall stiffening (Schopfer, 1996). As shown in this report, peroxidase is also able to degrade wall polymers and could, therefore, play an important role in the biologically controlled wall loosening responsible for turgor-driven cell growth and related physiological processes. In order to fulfill this function by producing $OH^{\cdot}$ directly from O_2 the enzyme must be provided with NADH, or an equivalent reducing substrate (Wojtaszek, 1997), in the cell wall. This requirement can be circumvented, however, if $O_2^{\cdot-}$ and H_2O_2 are supplied from other sources (see Eq. (4)). There is accumulating evidence that plant cells can secrete $O_2^{\cdot-}$ into the apoplast by a peroxidase-independent reaction at the plasma membrane (Vianello & Macrì, 1991; Ogawa, Kanematsu & Asada, 1997; Van Gestelen, Asard, Horemans & Caubergs, 1998). H_2O_2 can easily be produced from $O_2^{\cdot-}$ by dismutation and has been shown to accumulate in cell walls (Ogawa et al., 1997; Olson & Varner, 1993; Schopfer, 1994; Frahry & Schopfer, 1998). It is conceivable, therefore, that the mode of action of peroxidase in the cell wall can be controlled by the availability of these substrates from the protoplast. For substantiating this intriguing hypothesis it will be necessary to show first of all that peroxidase in fact functions in the $OH^{\cdot}$-producing mode in the living tissue.

3. Experimental

3.1. Chemicals

Peroxidase (EC 1.11.1.7; horseradish, type VI), pectin (apple) and dextran (*Leuconostoc mesenteroides*, av. mol. wt. 5×10^6–40×10^6) and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) were from Sigma, Deisenhofen, Germany. Superoxide dismutase (EC 1.15.1.1; bovine erythrocytes) was from Fluka, Deisenhofen, Germany. Xyloglucan (from Tamarind seeds), obtained from Dr. S.C. Fry (Edinburgh), was purified as described (Fry, 1998).

3.2. Assay of polysaccharide scission

The viscometric method described by Fry (1998) was used with minor modifications. Polysaccharide solutions (1 ml) in Na-citrate buffer (10 mM citrate, pH as specified) were incubated with various additives in 14-mm diameter vials at $25.0 \pm 0.2^{\circ}C$. Decrease in viscosity, indicative of scission of polysaccharide backbones, was determined at suitable time intervals as decrease in efflux time of 50 μl solution in a 100 μl glass pipette, the tip of which submerged in the test solution. The data represent means of at least 4 independent experiments ± s.e.

3.3. EPR spectroscopy

For determining the production of oxygen radical species 50 mM DMPO was added as a spin trap to reaction mixtures. Samples were filled into microcapillaries that were placed into conventional EPR tubes. EPR spectra were recorded with a Bruker ESR 200 X-band spectrometer operated at 9.44 GHz microwave frequency, 20 mW microwave power and 100 kHz modulation frequency.

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