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Cell suspension cultures of spruce (*Picea abies*): inactivation of extracellular enzymes by fungal elicitor-induced transient release of hydrogen peroxide (oxidative burst)

Burkhard Messner & Meinrad Boll
Abt. Zellchemie, G.S.F, National Center for Environment and Health, München, P.O.Box 1129, D-85758 Oberschleissheim

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Abstract

Elicitation of suspension culture cells of spruce [*Picea abies* (L.) Karst] with a fungal cell wall preparation of the spruce pathogenic fungus *Rhizosphaera kalkhoffii* Bubak induced inactivation of extracellular enzymes. Extracellular peroxidase, β-glucosidase and acid phosphatase, secreted by the cells during growth, and also α-amylase and pectinase from *Aspergillus* strains, added to an elicited cell culture, were inactivated. Inactivation is caused by an elicitor-mediated transient release of H_2O_2 from the cells (oxidative burst). H_2O_2 released into the medium was determined with ABTS (2,2'-Azino-bis-(3-ethylbenthiazoline-6-sulfonate)) (formation of blue colour) and with phenol red (destruction of pH indicator). The release started only minutes after beginning of elicitation and its inactivating effect existed for more than 1 day. Release of H_2O_2 is a biphasic process with a first smaller maximum at 1 h, followed by a second larger increase, peaking at 5–6 h and returning to approximately the control levels thereafter. Also H_2O_2 is transiently released in small quantities from cell incubations in the absence of elicitor as a stress response of the cells to manipulations of the cultures. Extracellular enzymes secreted into the medium could also be inactivated by direct addition of exogenous H_2O_2. Catalase prevents inactivation of the secreted extracellular enzymes, however, to a limited extent only because, as a result of contact of cells and medium, catalase becomes inactivated. The ionophores A 23187 and cycloheximide induced release of H_2O_2 and, when present together with elicitor, induction was synergistically increased.

Introduction

Plants can mobilize a large variety of different mechanisms in response to pathogenic attack. These are designed to either strengthen barriers against invasion or to weaken or even destroy the invading pathogen. The defense reactions include lignification (Kohle et al. 1984), suberization (Espelie et al. 1986), callose biosynthesis (Kohle et al. 1985), synthesis of antimicrobial phytoalexins (Kuc & Rush 1985) and induction of hydrolytic enzymes (Boller 1985). For a review see also Bell (1981). Lignification has been suggested in particular as a defense reaction in disease that is induced by fungal pathogens (Vance et al. 1980).

Another example of a defense strategy is the hypersensitive response (Doke & Ohashi 1988; Rogers et al. 1988). The hypersensitive reaction is expressed as rapid cellular death and tissue necrosis. The earliest detectable changes are electrolyte leakage (Goodman 1972), cell membrane depolarization (Pavlovkin et al. 1986) and lipid peroxidation (Keppler & Novacky 1986). This suggests a major alteration of membranes occurring in the initial phases of the hypersensitive response. The mechanism responsible for these alterations involves a transient production of active oxygen species (Adam et al. 1989; Apostol et al. 1989).

Systems of host-pathogen interactions, in which a role for active oxygen species has been documented, include potato (tuber and leaf tissue) and *Phytophthora infestans* (Chai & Doke 1987), rice leaf and *Pyricularia oryzae* (Sekizawa et al. 1987), soybean and *Verticillium dahliae* (Apostol et al. 1989), tomato suspension

cells and *Cladiosporium fulvum* (Vera-Estrella et al. 1992), tobacco and *Pseudomonas* sp. (Keppler et al. 1989) and spruce suspension cells and ectomycorrhizal as well as spruce pathogenic fungi (Schwacke & Hager 1992).

Relatively little is known concerning the mechanism of generating oxygen radicals as reponse to pathogenic attack. One possibility is that a specific NAD(P)H oxidase, located in the membranes, catalyzes a single electron reduction of molecular oxygen to O_2^- (Vianello & Macri 1989; Cross & Jones 1991). Dismutation of the superoxide anion produces H_2O_2. Peroxidases are also possible sources of oxygen radicals in plants (Mader & Amber Fisher 1982; Ramasarma 1982). Wall fractions of the plant cell are able to oxidize NAD(P)H and generate H_2O_2 and this activity is attributed to peroxidase (Elstner & Heupel 1976). Therefore, peroxidases have a dual function, i.e. production of H_2O_2 (Elstner & Heupel 1976) and polymerization in lignin biosynthesis (Moerschbacher et al. 1988; Messner & Boll 1993b) and are thus involved in both structural and physiological protection of the host.

In a previous communication (Messner & Boll 1994a), inactivation of extracellular enzymes in a suspension culture of spruce in response to elicitation with a cell wall preparation of *Rhizosphaera kalkhoffii* was described together with some properties of the inactivating factor. The objective of the present study was to further characterize an 'active oxygen' production (generation of H_2O_2) during the onset of the fungus-induced hypersensitive reaction. Part of this work has been published in preliminary form (Messner & Boll 1993a).

Materials and methods

Plant material

Cell suspension cultures

Seven-day-old photomixotrophic cell suspension cultures of *Picea abies* were used. Preparation of the cells from seedlings via callus cells and cultivation of the cells in Murashige and Skoog medium (1962) have been described in detail (Messner et al. 1991; Messner & Boll 1994a).

Fungal material and elicitor preparation

Rhizosphaera kalkhoffii was maintained on malt agar [30 g l^{-1} malt extract, 10 g l^{-1} agar (type A, Sigma)]. Preparation of the fungal cell wall fraction was performed as described previously (Messner et al. 1991).

Standard incubations

Cell cultures, grown for 7 days (Messner & Boll 1993b), were divided into Erlenmeyer flasks (300 ml total volume) that contained medium (either spent or fresh medium) plus cells (standard incubations). For experiments on inactivation of extracellular enzymes, 700 mg cells were contained in 30 ml medium (Messner & Boll 1993b, 1994a), while incubations involving H_2O_2 measurements were performed with 500 mg cells in 50 ml medium. Spent medium is designated as the culture medium of a 7-day-old suspension culture and it contains the extracellular enzymes secreted during this period. For incubations in spent medium the 7-day-old cell cultures in their medium were used directly. For incubations in fresh medium, the 7-day-old cells were separated from the medium by sedimentation and suspended in fresh medium. Fungal elicitor when present was 50 µg ml medium^{-1}. Incubations were with rotary shaking (50 rpm) at 27°C in continuous white light (Osram L 40W/77) (Messner & Berndt 1990).

Enzyme assays

Prior to determination of enzyme activity, the medium was cleared by centrifugation at 15 000x*g* for 3 min. Pectinase was assayed in the medium that had been washed by ultrafiltration (Messner & Boll 1994a). Enzyme assays, with the exception of pectinase (37°C), were performed at 25°C.

Enzyme activity was determined as follows: Peroxidase (EC 1.11.1.7) was measured with phenylenediamine as substrate (485 nm) (Schulz 1985; Ludewig et al. 1989). β-Glucosidase (EC 3.2.1.21) was assayed according to Marcinowski & Grisebach (1978) with 4-nitrophenylglucoside as substrate. Acid phosphatase (EC 3.1.3.2) was determined as described with 4-nitrophenylphosphate as substrate (Le Bansky et al. 1992). α-Amylase (EC 3.2.1.1) from *Aspergillus oryzae* was determined, using amylopectin azure (Sigma) as substrate, according to Wahlefeld (1974) with a minor modification; colour in the incubation mixture (0.5 ml) was determined after unhydrolyzed amylopectin azure was precipitated with 0.5 ml of isopropanol. Pectinase (EC 3.2.1.15) from *Aspergillus*

niger was determined with polygalacturonic acid as substrate and determination of the galacturonic acid released with dinitrosalicylic acid (Messner & Boll 1994a).

Activity is expressed as follows: Peroxidase, ΔE 485 nm min^{-1} mg $protein^{-1}$ (Schulz 1985; Ludewig et al. 1989); activity of the other enzymes is expressed as nKat mg $protein^{-1}$. Protein was determined according to Bradford (1976), using bovine serum albumin as standard.

Assay of hydrogen peroxide

ABTS: The concentration of H_2O_2 present at a definite time during elicitation was determined as described (Bergmeyer & Bernt 1974) with some modifications: cells were cultivated for 7 days. They were then carefully washed twice with fresh medium and suspended in fresh medium. The suspension was adjusted to 500 mg cells/50 ml medium. Elicitor was added at time zero. At the indicated times, a 1.2 ml aliquot was removed from the suspension and the cells were separated from the medium by filtration. For H_2O_2 determinations to 1 ml of medium 0.1 ml 0.1 M KH_2PO_4, 0.01 ml horseradish peroxidase (1 mg ml^{-1}, dissolved in medium) and 0.01 ml ABTS solution (0.05 M) were added. Absorbance at 415 nm was measured after 3 min. The H_2O_2 standard consisted of a freshly prepared solution, whose concentration was determined at 240 nm (e_{240} = 0.040 cm^2/mmol). The ABTS assay could not be used in spent (7-day old) medium since phenolic substances formed during this time interfere with the formation of the blue colour.

Phenol red: The total amount of H_2O_2 released from the initiation of elicitation was measured as described (Grisham et al. 1984) with some modifications: cells were cultivated, washed and suspended as described above. Subsequently to a flask containing 500 mg of cells in 50 ml medium,1 ml of phenol red (2 mM) and 0.5 ml horseradish peroxidase (1 mg ml^{-1}) (both dissolved in medium) were added. Phenol red in the concentration employed is not toxic to the cells and is neither taken up nor metabolized by the cells. Elicitor was added at time zero. At the indicated times 1 ml aliquots were removed, 0.02 ml of 0.5N NaOH was added and the extinction was immediately measured at 558 nm. Extinction decreases in the presence of H_2O_2, the extent being proportional to the amount released. Linearity is limited to 60–70 percent of the dye present at the beginning. Increasing distance to the initial extinction value is measured. The H_2O_2 standard was the same as above.

Biochemicals

ABTS was purchased from Boehringer, Mannheim, Germany. All other biochemicals, including enzymes, were obtained from Sigma Chemical Co., St.Louis, USA, (Deisenhofen, Germany).

Results

Inactivation of extracellular enzymes

Several enzymes are secreted into the medium in increasing quantities during growth of the spruce cells in culture. These include extracellular peroxidase, β-glucosidase and acid phosphatase (Messner & Boll 1994a). After 7 days of cultivation of the suspension cells, specific activity of the enzymes in the medium was as follows: peroxidase, 850 ± 76 ΔE min · mg $protein^{-1}$; β-glucosidase, 4.5 ± 0.65 nKat mg $protein^{-1}$ and acid phosphatase, 410 ± 33 nKat mg $protein^{-1}$. When the 7-day-old cell culture is elicited with *Rhizosphaera kalkhoffii*, a number of defense reactions are induced in the cells, e.g. induction of lignin synthesis (Messner & Boll 1993b). The secreted extracellular enzymes become inactivated under these conditions. Thus, in response to elicitation, the spruce cellular peroxidase activities become induced (Messner & Boll 1993b), while the extracellular activities are inactivated (Messner & Boll 1994a).

Inactivation is not restricted to extracellular enzymes secreted by the spruce cells, but foreign enzymes, as e.g. lytic enzymes from fungi, α-amylase and pectinase, are also inactivated. In Fig. 1, inactivation is summarized. The decline in activity of all enzymes is substantial. Kinetics are similar for acid phosphatase, β-glucosidase, α-amylase and for pectinase, being slower at the beginning of elicitation than inactivation of peroxidase. The reason for the different kinetics might be a different sensitivity of the enzymes to the inactivating factor. Under identical experimental conditions the extent of inactivation could vary from one experiment to the other. However, peroxidase was always most substantially inactivated.

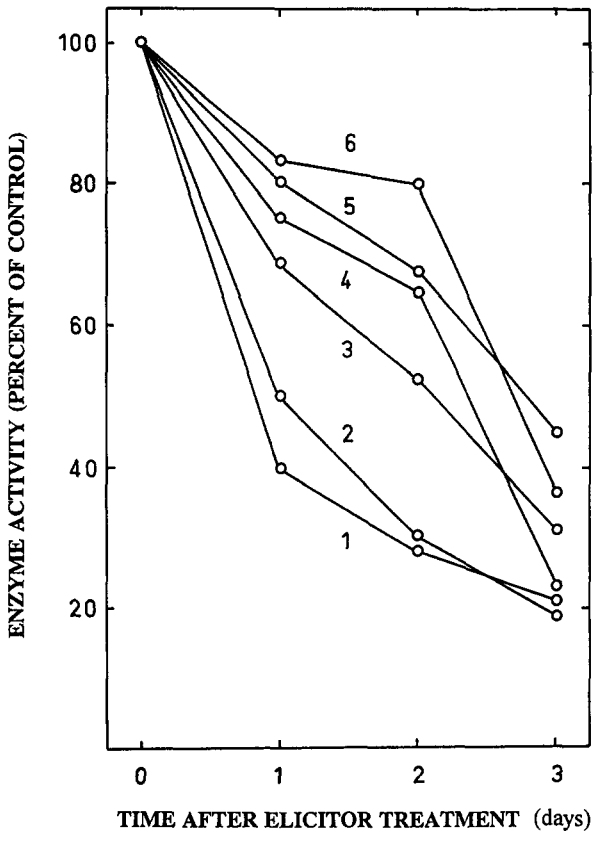

Fig. 1. Inactivation of extracellular enzymes in spruce cell suspension cultures in response to elicitation with a fungal cell wall preparation. Standard incubations (700 mg/30 ml) were made in spent medium, containing the extracellular peroxidase, β-glucosidase and acid phosphatase secreted during the 7-day cultivation period. Horseradish peroxidase, α-amylase and pectinase were incubated in fresh medium. Enzyme activities added per 30 ml medium : horseradish peroxidase 9.9 mg (3.3 mKat mg^{-1}), α-amylase 1 mg (containing 660 nKat) and pectinase 0.032 mg (containing 22 nKat). Elicitor was added at time zero. Each point represents determination of activity in three separate flasks. Values are percent of specific activity of controls (standard incubations without elicitor). SEM (6–13 percent) omitted for clarity. (1) horseradish peroxidase; (2) extracellular peroxidase; (3) α-amylase; (4) acid phosphatase; (5) pectinase; (6) β-glucosidase.

Properties of the inactivating factor

The inactivating factor originates in the cells after contact of the cells with the fungal cell wall fraction (elicitor). Its appearance was very rapid as shown by an immediately starting inactivation (Fig. 1). The inactivating effect apparently is unspecific since inactivation occurs with a number of quite different secreted extracellular enzymes and also with lytic fungal activities (Fig. 1). The inactivating factor is unstable. As shown in Fig. 1 the factor,in addition to inactivating secreted extracellular peroxidase, also inactivated commercial horseradish peroxidase added to the medium. However, addition of this enzyme at different times after beginning of elicitation led to a drastically reduced inactivation, an indication that the factor gradually looses its inactivating capacity (Messner & Boll 1994a). The inactivating factor is not a protease. Also an inactivating effect of phenolic compounds is unlikely (Messner & Boll 1994a).

Elicitor-induced release of active oxygen species

Thus, the inactivating effect might be considered a burst of destructive activity, identical with the so-called oxidative burst, the pathogen-induced formation of transiently active oxygen species.

Cell suspension cultures of spruce responded to fungal elicitation by immediately initiating the production of H_2O_2, which appeared in the medium only minutes after treatment with elicitor (Fig. 2). The H_2O_2 released reached a maximum and then declined again (ABTS) (Fig. 2A and B, curves 4). The decline was perhaps in part due to degradation of the released H_2O_2. Total amount of H_2O_2 (phenol red) increased until the release slowed down between 4 and 7h (Fig. 2B, curve 2). The value of 24h was beyond the linearity of the phenol red determination. However, in contrast to other studies of plant systems that reported a maximum release of H_2O_2 within an hour after beginning of elicitation, the maximum here was not reached before 5 h. A first small increase peaking at 60 min was followed by a depression at 90–120 min and increase resumed after about 150 min (Fig. 2A and B, curves 4). While the peak point of the two maxima of H_2O_2 release does not change, amounts released could vary among the experiments (see also Table 3).

An additional feature of the process is the transient release of H_2O_2 that occurs in the absence of elicitor (Fig. 2A, curves 1 and 3). This increase, although much smaller than with elicitor present (Fig. 2A, curves 2 and 4), was significant and it followed the same kinetics as in the incubations containing the elicitor. However, when cells were incubated for 24 h and elicitation was then initiated (Fig. 2B), release of H_2O_2 in the elicitor-free controls was negligible (Fig. 2B, curves 1 and 3). Therefore, the transient increase of H_2O_2 in the former incubations can be designated to be the response reaction of the cells to transfer of the culture into fresh medium. Such stress response is a widespread phenomenon in secondary metabolism of plant

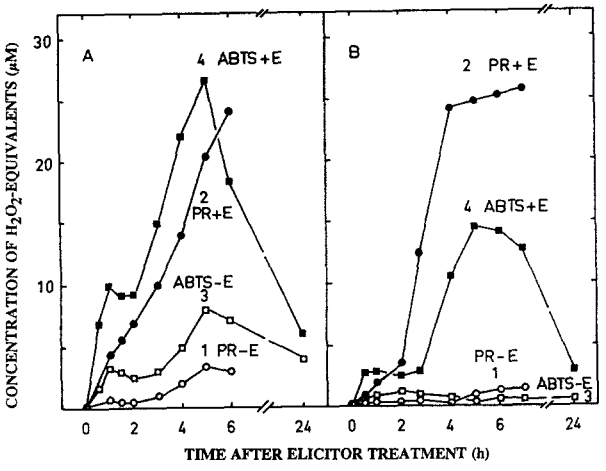

Fig. 2. Time course of release of hydrogen peroxide from elicitor-treated spruce suspension culture cells. Standard incubations (500 mg cells/50 ml) were made in fresh medium and H_2O_2 was determined as described. Values represent determination in 3 separate incubation flasks. SEM (5–12 percent) omitted for clarity. (*A*) Medium plus cells. Elicitor was added immediately (time zero). (*B*) Medium plus cells. Samples were incubated for 24 h. Elicitor was then added (time zero). (1) (PR-E) phenol red, no elicitor; (2) (PR+E) phenol red, plus elicitor; (3) (ABTS-E) ABTS, no elicitor; (4) (ABTS+E) ABTS, plus elicitor

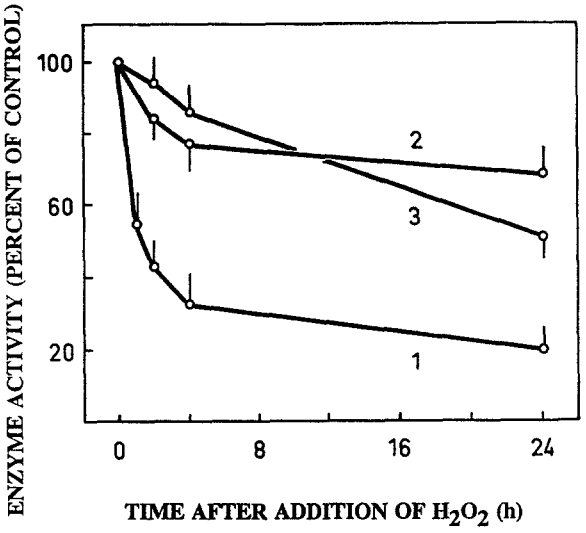

Fig. 3. Inactivation of extracellular enzymes of spruce cell suspension cultures by exogenous hydrogen peroxide. Spent medium, containing the extracellular enzymes secreted during 7 days of cultivation was used. Incubations were 30 ml of this spent medium without cells or elicitor. Inactivation of the extracellular enzymes was initiated by addition of 2 mM H_2O_2 (time zero). Values are percent of the specific activity of controls (medium without elicitor) ± SEM. (1) extracellular peroxidase; (2) β-glucosidase; (3) acid phosphatase

cell cultures (Hahlbrock & Wellmann 1973; Rolfs et al. 1987; Messner et al. 1991).

Inactivation of extracellular enzymes by exogenous hydrogen peroxide

Peroxidase, β-glucosidase and acid phosphatase, which are secreted into the medium during growth of the cells and which are inactivated on elicitation, also became inactivated by exogenous H_2O_2 (Fig. 3). As shown for the inactivation induced by the fungal elicitor, peroxydase again is most substantially inactivated and β-glucosidase and α-amylase also exhibited inactivation kinetics different from those of peroxydase (compare Figs 1, 3). Because of the higher H_2O_2 concentrations present, inactivation of the enzymes is considerably more pronounced then it is in response to elicitor-released H_2O_2 (see Fig. 1). Inactivation depended on the concentration of the added H_2O_2. There was, however, no true dose-response effect (see Discussion).

Effect of catalase

As shown in Table 1, catalase prevented inactivation of peroxidase (taken as representative enzyme), however, to a limited extent only. Significantly increasing the catalase concentration will only slightly increase the capacity of the enzyme to diminish inactivation of peroxidase (Table 1). The reason for the incomplete action of catalase on the H_2O_2-mediated inactivation of peroxidase very likely is an inactivation of cata-

Table 1. Effect of catalase on the elicitor-induced inactivation of peroxidase.[1]

Catalase (μg ml^{-1} medium)	Peroxidase activity (%)	
	1 day	2 days
0	52.4 ± 3.1	32.2 ± 2.7
8.8	58.2 ± 4.0	40.0 ± 3.3
17.6	62.1 ± 4.8	48.2 ± 3.8
35.2	68.8 ± 4.6	53.4 ± 4.2

[1]Standard incubations (300 mg/30 ml) were made using spent medium (containing extracellular peroxidase and the other extracellular enzymes of the 7-day cultivation period). Different concentrations of catalase (specific activity 25,000 units mg protein^{-1}) were added to the samples prior to elicitor. Elicitor was added at time zero. Peroxidase activity was determined after 1 and 2 days of elicitation in three separate incubations. Values are percent of specific activity of controls (incubation without elicitor) ± SEM.

lase as a consequence of an interaction of medium and suspension cells (Table 2). H_2O_2 when added to fresh medium could very rapidly be removed completely by catalase (A). When, however, the medium was incubated with suspension cells and, after separation of cells and medium, H_2O_2 was then added, it could only in part be eliminated by catalase. The capacity of catalase decreases with increasing time of medium and cells being incubated (B-F). Incubation of only 2 h already results in a drastic reduction of catalase activity (B).

The H_2O_2 levels at time zero of Table 2 (beginning of catalase action (II)) are different since they consist of the added H_2O_2 (20 µM) (A) plus the amount that was transiently released as the stress response during incubation (B-F). The stress-released H_2O_2 had a maximum at around 6 h (D) with the same kinetics as shown in Fig. 1A, curve 3. Despite the fact that the initial concentrations of H_2O_2 differed, with the excess of catalase present, this did not affect determination of the decrease of H_2O_2.

Effect of ionophores

The effect of two ionophores, A 23187 and cycloheximide, on the formation of H_2O_2 by suspension culture cells is shown in Table 3. Release of H_2O_2 was determined in a cell culture that had been preincubated for 1 day to exclude an additional increase of H_2O_2 due to the stress response. In the presence of the ionophores release of H_2O_2 is significant (compare A and C), although less substantial than with elicitor (compare A and B). Treatment of the cells with ionophore plus elicitor resulted in a synergistically increased secretion of H_2O_2 (compare B,C and D). The secretion of H_2O_2 by treatment with ionophore also was time-dependent, its maximum being after about 6 h of incubation, as with the elicitor-induced H_2O_2 formation (Fig. 2).

Discussion

The elicitor exerts two different effects on the spruce cell suspension culture. It induces formation of lignin and also inactivation of extracellular enzymes. Despite inactivation of extracellular peroxidase, the so-called lignin peroxidase (Castillo 1986), nevertheless lignin material is formed in the medium (Messner & Boll 1993b). Since the velocity of the peroxidase reaction is highly dependent on the amount of H_2O_2 present, the polymerization reaction will depend mainly on this H_2O_2 concentration. It might well be that the H_2O_2 released by treatment with elicitor will lead to an increased polymerization reaction in lignin biosynthesis. This result would imply that the activity of the extracellular peroxidase, which becomes lowered by the elicitor, is still sufficient for this process.

The elicitor-mediated inactivation of extracellular enzymes, as it occured here, probably resulted from the oxidative burst, being one of the steps towards necrosis. Effects produced by the elicitor will depend on its momentary concentrations (Messner et al. 1991). Low concentrations with a small release of H_2O_2 will increase protein synthesis and lead to induction of enzymes (Messner & Boll 1993b). Higher concentrations of elicitor, resulting in higher quantities of H_2O_2 released, will eventually cause the cells to become necrotic.

When comparing details of the oxidative burst of the *Picea abies* cell culture by an elicitor from *Rhizosphaera kalkhoffii* with other values in the literature, it apparently has a maximum of H_2O_2 release that is higher and that also is reached later during elicitation. A maximum release at around 25 min (Keppler et al. 1989; Schwacke & Hager 1992; Orlandi et al. 1992) or at 60–90 min (Murphy & Huerta 1990) has been reported. Peak concentrations of released H_2O_2 were 8–9 µM (Murphy & Huerta 1990) and 5 µM (Schwacke & Hager 1992). The oxidative burst described here obviously is a biphasic process and the first smaller increase of H_2O_2, peaking at 50–60 min with 4–7 µM H_2O_2 released (Fig. 2), might correspond to the values reported elsewhere. Since the other investigations, in contrast to the present study, have not been performed under true conditions of cell cultivation, the kinetics of H_2O_2 release could not be followed beyond periods of more than a few hours. Kinetics similar to those reported here have been reported once (Orlandi et al. 1992). Possibly the first smaller peak of H_2O_2 secretion (50–60 min) is the oxidative burst as it is described by others, while the second larger increase (5–6 h) represents secretion of H_2O_2 as fungitoxic substance that ultimately leads to necrosis of the cells. Also this H_2O_2 could serve as substrate for lignin biosynthesis.

In all plant-pathogen systems studied, the release of active oxygen species reaches a maximum and declines thereafter. Two possibilities for the decrease of H_2O_2 concentration might be discussed. Either a degrading system is established or, more likely, the production of H_2O_2 is terminated and the H_2O_2 still present becomes degraded by the constitutive peroxidase (Schwacke & Hager 1992).

Table 2. Effect of incubation of spruce suspension cells in medium on the activity of catalase.[1]

	I Incubation (hours)	II H_2O_2 (μM)	III H_2O_2 (percent of initial concentration)		
			5'	15'	30'
A	0	19.4 ± 1.29	10.5 ± 1.04	5.2 ± 0.48	3.1 ± 0.42
B	2	24.2 ± 2.15	50.6 ± 5.1	38.4 ± 3.4	22.1 ± 2.18
C	4	26.1 ± 2.54	56.2 ± 4.9	45.1 ± 4.7	32.6 ± 3.15
D	6	27.1 ± 2.18	64.6 ± 6.1	52.1 ± 4.9	47.2 ± 4.58
E	8	24.5 ± 2.21	69.3 ± 6.4	57.4 ± 5.2	50.1 ± 5.23
F	24	19.9 ± 1.15	90.3 ± 8.9	80.3 ± 7.9	69.2 ± 7.03

[1]The cells of a 7-day-old suspension culture were washed twice with fresh medium. Then to 30 ml each of fresh medium 300 mg of the washed cells were added and the samples incubated (I) for 0 h, 2h, 4 h, 6 h, 8 h and 24 h. In the unicubated control sample the cells, after addition to the medium, were removed again immediately. At the indicated times, medium and cells were separated by filtration. To 20 ml medium of each sample, 20 μl H_2O_2 (20 mM) was added and total H_2O_2 concentration in the different samples was determined (ABTS) (II). Then 2 mg catalase (specific activity 25,000 units mg protein^{-1}) were added per sample (time zero). The samples were then incubated and $H_2O_2$was measured after 5, 15 and 30 min (III). Values are percent H_2O_2 of the initial concentration in three separate incubations ± SEM.

Table 3. Effect of ionophores on the release of H_2O_2 from elicitor-treated spruce suspension culture cells.[1]

	Incubation	μM H_2O_2 released after 6 hours	
		A 23187 5×10^{-6} M	cycloheximide 10^{-5} M
A	control	0.21 ± 0.030	0.24 ± 0.027
B	+ elicitor	9.43 ± 0.89	4.51 ± 0.51
C	+ ionophore	4.11 ± 0.44	2.85 ± 0.30
D	+ elicitor + ionophore	22.19 ± 2.17	36.41 ± 3.71

[1]Standard incubations were made in fresh medium (500 mg cells/50 ml medium). Elicitor and/or inophore were added at time zero. A 23187 was dissolved in DMSO (5 mg in 0.95 ml) and 25 μl were added per 50 ml. Cycloheximide was dissolved in water. Controls showed no effect of DMSO on the activity of the cells. Incubations were made for 6 h H_2O_2 was determined with the ABTS method.

Two lines of evidence have been presented showing that the released H_2O_2 is the responsible effector for the decrease of the extracellular enzymes. The enzymes become inactivated also by added exogenous H_2O_2 and catalase can prevent the inactivation. Inactivation by H_2O_2 is not dose-dependent over the entire concentration range tested. Dose dependency was found between 10 and 50 μM added H_2O_2. Above this concentration (up to 2 mM) the extent of inactivation by H_2O_2 increases only gradually. Possibly in response to the action of H_2O_2 the resulting oxidized form of the extracellular enzymes will still contain some residual activity. Estimation of very small amounts of H_2O_2 (below 5 μM) was found to become increasingly less accurate as the 7-day-old (spent) medium probably contains a number of substances (phenolics) capable of gradually destroying H_2O_2. The experiment of Fig. 2 was performed in fresh medium that, of course, does not contain substances that could destroy the H_2O_2.

When comparing inactivation of the extracellular enzymes by elicitor-induced H_2O_2 formation and by added exogenous H_2O_2, it should be noted that in the latter case inactivation is measured with the total amount of H_2O_2 present at the beginning of the experiment, while in the former case inactivation was the result of a gradually increasing release of H_2O_2 from the elicited cells. It cannot be excluded that during elicitation, other oxygen species are also released into the medium which might lead to inactivation. Thus, inactivation by H_2O_2 as it is observed could be smaller.

As mentioned above catalase can only partly prevent the H_2O_2-mediated inactivation, probably

because the enzyme becomes increasingly inactivated when cells are incubated in medium (Table 2). Thus, during incubation of suspension cells either an inhibitory substance is excreted from the cells or the medium is altered in some way by the cells. The possibility that during mere incubation of the cells an inhibitor specific for catalase is produced is rather unlikely. If one considers changes of the ionic environment of the medium caused by the cells, which are detrimental to catalase activity, this possibility is more likely. Protease as an agent for the inactivation of catalase can be excluded since no protease activity could be detected in the medium containing elicited cells (Messner & Boll 1994a).

The mechanism underlying the inactivation remains to be evaluated. It should be noted that inactivation is still seen despite the elicitor-induced release of H_2O_2, after having passed through the maximum, is nearly terminated (compare Figs 1, 2). This result would require a separate mechanism for the inactivation or an indirect effect, mediated through the H_2O_2 present during the initial phases of the inactivation process. This mechanism would still have to be operating after H_2O_2 has disappeared and lead to continuation of inactivation. Inactivation of enzymes by H_2O_2 and metals (Fe, Cu, Mn, etc.) has been described in detail and a mechanism for such metal-catalyzed oxidation has been proposed (Stadtman & Oliver 1991). As the result of the oxidative modifications, the enzyme protein becomes absolutely sensitive to proteolytic degradation and is converted to catalytically less active or inactive forms (Oliver et al. 1987). However, it is not clear whether inactivation of peroxidase, β-glucosidase and of acid phosphatase, as shown in Fig. 1, will go to completeness, since the activities, after having reached a certain minimum level, resumed to increase again (Messner & Boll 1994a). This renewed increase was a result of the secretion of these enzymes into the medium that continued during elicitation and it would compensate inactivation during later phases of the process.

Inactivation of pectinase occurring in response to elicitation with *Rhizosphaera* (Fig. 1) is only part of a more complex series of effects (Messner & Boll 1994a). Pectic polysaccharides (oligogalacturonides) are a class of elicitors released from plant cell walls. These can act alone as well as synergistically with pathogen-induced elicitors (Davis et al. 1986). The following sequence of events could be visualized here: pectinase attacks the spruce cells resulting in the formation of an elicitor which is released from the cell walls. The *Rhizosphaera* wall fraction reacts with the spruce cells as an elicitor. The two elicitors give rise to the same effector, H_2O_2. The presence of H_2O_2 then leads to the effects observed in the cell culture, i.e. formation of lignin from its monomers (coniferylalcohol) in a peroxidase reaction and inactivation of extracellular enzymes. Accordingly pectinase becomes inactivated by the H_2O_2 induced via the cell wall elicitor and inactivation is intensified by the H_2O_2 released by the fungal elicitor (Messner & Boll 1994a). Thus, pectinase, by merely attacking the spruce cell walls, turns on its own inactivation.

H_2O_2 as possible effector of elicitation by pectinase was established when preparing protoplasts from the spruce cells (Messner & Boll, et al. 1994b). In response to addition of pectinase (contained in the protoplast isolation mixture), increasing amounts of H_2O_2 were released from the cell preparation (ABTS determination). In the presence of ascorbic acid, which removes H_2O_2, formation of protoplasts was significantly faster and the yield of protoplasts was much higher. Thus, ascorbic acid apparently protects pectinase from being inactivated by H_2O_2.

Ca^{2+} is suggested to participate in initiating plant defense mechanisms (Atkinson et al. 1990) and the influx of Ca^{2+} apparently is a critical point in elicitor-induced signal transduction (Stäb & Ebel 1987; Waldmann et al. 1988; Atkinson et al. 1990). Elicitor-induced formation of active oxygen was found to be reduced in a Ca^{2+}-depleted cell culture medium (Schwacke & Hager 1992). The possibility of K^+ efflux and polarization of the membranes has also been discussed (Atkinson et al. 1985; Pelissier et al. 1986; Conrath et al. 1991). Recently a cycloheximide-induced K^+ efflux has been measured and described (Murphy 1988). As the result of this study an enzyme protein with a rapid turnover, responsible for the accumulation of K^+ in the cells is postulated whose synthesis is blocked by cycloheximide, allowing efflux of K^+ to occur. Alternatively reduction in protein synthetic activity (by cycloheximide) might stimulate the formation of a specific compound that induces efflux of K^+ (Murphy 1988). A ionophoric effect of cycloheximide seems possible here since the elicitor-induced release of H_2O_2 does not necessarily include protein synthesis. In contrast elicitor-induced protein synthesis, e.g. enzyme induction for lignin synthesis, is well prevented by cycloheximide (Messner & Boll 1993b).

The results presented here could indicate that Ca^{2+} influx is stimulated by the ionophore A 23187. Also efflux of K^+ is stimulated by cycloheximide (Table

3). The fact that H_2O_2 secretion is increased in control (non-elicited) incubations and also in elicited cell incubations would suggest that both increase of cellular Ca^{2+} level and decrease of cellular K^+ level have a stimulatory effect on the basal as well as on the elicitor-induced level of the H_2O_2-forming activity. Together with the observation that the synergistically increased release of H_2O_2 occurs rather late during treatment (not rapid as would be expected for signal transduction), this might indicate that the cellular levels of the two ions in this system not only function as a second messenger but also as an activator of the H_2O_2-forming system. In order to verify these functions of Ca^{2+}, a correlation would have to be established, employing protoplasts, between the amount of Ca^{2+} entering the cell and the quantity of the H_2O_2 that becomes released.

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