A method to detect oxidative stress by monitoring changes in the extracellular antioxidant capacity in plant suspension cells

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Abstract

Detection of H$_2$O$_2$ in the supernatant of plant suspension cells is often used to indicate the time and extent of the oxidative burst during interactions with either bacteria or pathogen-related elicitors. We have found that suspensions of plant cells, depending on conditions, may produce considerable levels of extracellular phenolics that can function as antioxidants and prevent or suppress the detection of H$_2$O$_2$. These compounds can be used as substrates by extracellular peroxidases to scavenge stoichiometric amounts of H$_2$O$_2$. When this occurs during plant/pathogen interactions it can mask both the timing and extent of the oxidative burst if detection of free H$_2$O$_2$ is the only technique used. We have developed a chemiluminescent technique that will account for the H$_2$O$_2$ scavenged by these extracellular metabolites. A known quantity of H$_2$O$_2$ is added to samples and allowed to react with the extracellular antioxidants. The amount of H$_2$O$_2$ that remains is then determined by adding luminol to the sample and measuring luminol-dependent-chemiluminescence. The difference between treated and control samples represents the amount of H$_2$O$_2$ that has been produced by the cells in response to the treatment. We have found that this technique provides a better estimate of both the magnitude and timing of the oxidative burst in bacterial/suspension cell systems.

Keywords: Reactive oxygen; Hydrogen peroxide; Pseudomonas syringae; Nicotiana tabacum L. cv. Hicks; Solanum tuberosum cv. Kennebec; Phenolics

1. Introduction

Plant/bacterial model systems incorporating suspension cells continue to provide unique insights into plant pathogenic interactions [4–6, 8–10]. They have been especially valuable in gaining a better understanding of the role of oxidative metabolism in pathogenesis where reactive oxygen species and other metabolites are often transient and/or difficult to detect. For many years the oxidative burst has been detected by assaying for the appearance of H$_2$O$_2$ using sensitive chemiluminescent or fluorescent assays that linked directly to the free H$_2$O$_2$ present in the samples.[11–13,15] Recently we found that suspensions of plant cells that are transferred to fresh assay buffer, produce substantial levels of extracellular antioxidants that can attenuate and often completely mask the oxidative burst in plant cells treated with bacterial pathogens [2]. It appears that as the oxidative burst occurs, the H$_2$O$_2$ associated with the event is continually scavenged by the cell wall peroxidases using the extracellular antioxidants as reductants. Unlike the cytoplasmic antioxidant mechanisms, which have considerable reserves of NADH or NADPH to regenerate antioxidants, the extracellular antioxidant mechanisms appear to be limited to the finite extracellular pool of ascorbic acid and phenolic antioxidants. The ascorbic acid in the cell wall region is capable of regenerating oxidized phenolics. There is evidence that the oxidized form of ascorbic acid, dehydroascorbate, is shuttled back to the cytoplasm and regenerated, however, this is a time dependent process [16,17,19,20].

We have developed a new technique that, (1) quantifies the constitutive and induced production of extracellular...
antioxidants by cell suspensions, and (2) provides a more complete estimation of oxidative events in suspension cells by accounting for reactive oxygen that has been scavenged by the extracellular antioxidants. In brief, a known amount of exogenous H$_2$O$_2$ is added to samples, allowed to react with the available extracellular antioxidant, and finally the amount of H$_2$O$_2$ that remains is determined. In untreated or control samples, the exogenous H$_2$O$_2$ consumed is directly related to the amount of extracellular antioxidant present. In treated samples that have undergone an oxidative burst, a portion of the extracellular antioxidants will have been oxidized and less exogenous H$_2$O$_2$ will be consumed by these samples. We have found this assay to give a more accurate estimation of the magnitude of the oxidative response of plants cells to pathogens and pathogen-related elicitors.

2. Materials and methods

2.1. Chemicals

Horseradish peroxidase (P-8250, purchased in units; one unit will oxidize 1 µmol of 2,2’-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) min$^{-1}$ as described by Sigma), guaiacol (G-5502), hydrogen peroxide (H1009) and luminol (5-amino-2,3-dihydro-1,4-phenathazinedione, A8511) were purchased from Sigma-Aldrich Chemicals Inc. (St Louis, MO, USA).

2.2. Plant material

Suspension cells of tobacco (Nicotiana tabacum L. cv. Hicks) and potato tuber (Solanum tuberosum cv. Kennebec) were maintained as previously described [2] except that tobacco callus and suspension cells were maintained on MS media (supplemented with 200 mg l$^{-1}$ KH$_2$PO$_4$, 0.2 mg l$^{-1}$ 2,4-D and 0.1 mg l$^{-1}$ kinetin). Tobacco cultures, 10 ml, were transferred to 80 ml fresh media every 4 days. Potato cultures, 13 ml, were transferred to 80 ml fresh media every 7 days. Routinely, 2-day-old tobacco cells and 5-day-old potato cells were used for experiments. Cells were washed and suspended in assay buffer, containing 0.5 mM CaCl$_2$, 0.5 mM K$_2$SO$_4$, 175 mM mannitol and 0.5 mM MES (pH 6) with a final cell density of 0.05 g ml$^{-1}$. Cell suspensions, 25 ml, contained in 50 ml beakers were equilibrated for 0.5 h in a rotary water bath shaker set at 27 °C and 180 rpm for tobacco cells or 200 rpm for potato cells. Treatments were added directly to the suspensions. All experiments were preformed twice with at least two replicates per treatment.

2.3. Bacterial preparations

Cultures of Pseudomonas syringae pv. syringae 61 were maintained as previously described [1]. Isolate WT (HR $+$) causes a hypersensitive reaction when infiltrated into tobacco and potato leaves. Isolate B7 (HR $-$) is a Tn5 insertion mutant that does not induce a hypersensitive response [3]. Bacterial cultures were grown for 20 h in Kings B broth, centrifuged, washed and suspended in deionized water. Based on optical density, the concentration of the suspension was adjusted with water so that addition of about 200 µl of the bacterial suspension to plant cell suspensions would result in $2 \times 10^7$ cfu ml$^{-1}$. Bacterial concentrations in cell suspensions were verified periodically by dilution plating.

2.4. Extracellular hydrogen peroxide assay

The luminol-dependent-chemiluminescent assay was used to detect extracellular H$_2$O$_2$ [11]. Using a wide-bore pipette, 0.45 ml samples of treated or untreated suspension cells were dispensed into tubes, and placed into a EG&G Berthold Autolumat 953 luminometer (Bad Wildbad, Germany). A stock solution was prepared containing horseradish peroxidase, 28.8 units ml$^{-1}$, and luminol, 1.7 mM, in 50 ml of 1 M sodium phosphate, pH 7. As each sample was ready to be assayed, the luminometer added 50 µl of the stock solution for a final concentration of 2.88 U ml$^{-1}$ of peroxidase and 170 µM luminol. Chemiluminescence was recorded immediately as relative light units (RLU) every 0.1 s for 20 s. Under the standard conditions described here the peak height is proportional to the concentration of H$_2$O$_2$ in the sample. Standard curves were prepared with dilutions of H$_2$O$_2$ in assay buffer.

2.5. Extracellular antioxidant assay

The extracellular antioxidant capacity of cell suspensions was estimated by determining the amount of exogenous H$_2$O$_2$ consumed by samples. Because the antioxidant mechanism is rapid under the conditions described, it could be carried out using a modification of the luminol-dependent-chemiluminescent assay described above. A stock reagent of H$_2$O$_2$ contained 200 or 500 µM H$_2$O$_2$ in assay buffer for potato or tobacco samples, respectively. The H$_2$O$_2$ reagent, 50 µl, was automatically added to suspension samples, 0.4 ml, in the luminometer 4.5 s prior to the peroxidase/luminol reagent, 50 µl. This extra time was sufficient for the H$_2$O$_2$ to react with endogenous cell wall peroxidase and the extracellular antioxidant. The amount of extracellular antioxidant can be estimated by calculating the difference in RLU between controls and treatments. Standard curves were prepared with dilutions of H$_2$O$_2$ in assay buffer.

When this assay is applied to an untested cell line, preliminary assays with untreated control cells need to be conducted. First, the amount of exogenous H$_2$O$_2$ that is required to completely oxidize the extracellular antioxidants needs to be determined empirically. We have found it best to have at least 10 µM H$_2$O$_2$ in excess at the end of
the monitoring period. If the exogenous H2O2 is completely consumed the amount of antioxidant will be underestimated.

2.6. Endogenous extracellular peroxidase assay

Much of the extracellular peroxidase activity of these cell suspensions was associated with the cell wall. This assay attempts to estimate the total extracellular peroxidase activity in cell suspensions. Using a wide-bore pipette, 0.5 ml samples of cell suspensions were added to beakers containing: 9.1 ml sodium phosphate buffer, 50 mM pH 6; 0.2 ml guaiacol, 0.4 mM; 0.2 ml H2O2, 0.4 mM. The reaction mixtures were shaken in a water bath, 27°C, for a 15 min period. Aliquots were removed periodically, filtered through miracloth to remove particles, and their absorbance determined on a Beckman DU 650 spectrophotometer at 470 nm. The linear rate of increase in absorbance, OD470 min⁻¹, was used to monitor changes in peroxidase activity over time.

3. Results and discussion

A previous study demonstrated that cell suspensions of tobacco and potato produced extracellular phenolics that were able to react with H2O2 using cell wall bound peroxidase [2]. We will first show examples of how the technique reported here can be used to quantify both the production of these phenolics as well as the oxidative events that oxidize these phenolics. Subsequently we describe the critical parameters that should be considered in using this technique or adapting it to other cell systems.

3.1. Monitoring antioxidant capacity during tobacco/bacterial interactions

Changes in the extracellular antioxidant capacity of tobacco suspension cells either untreated or treated with *P. syringae* pv. *syringae* strains were followed over a 6 h period (Fig. 1). Hydrogen peroxide, 50 µM, was added to samples to react with the extracellular antioxidants and subsequently, additional peroxidase and luminol were added to react with the remaining H2O2. The peak chemiluminescence was recorded and by comparison to a standard curve, the approximate amount of H2O2 that had reacted with the extracellular antioxidant was determined (Fig. 1A). Therefore, as the extracellular antioxidants increase and react with more of the added H2O2, the chemiluminescence decreases. Untreated tobacco cells began accumulating extracellular antioxidants after about 1.5 h and by 6 h the concentration was sufficient to react with nearly 35 µM H2O2. Treatment of the tobacco cells with strain B7 (HR⁻), 0.2×10⁷ cfu ml⁻¹, increased the antioxidant capacity to nearly 40 µM H2O2 by 6 h, slightly more than untreated cells. We have found that this induction of extracellular phenolics varies with the bacterial concentration (unpublished data). Cells treated with strain WT (HR⁺) behaved similarly to strain B7 (HR⁻) until about 4 h when the antioxidant capacity decreased and became negative (prooxidative) by 5–6 h. This indicated that H2O2 was being produced and by 5 h it reached a level greater than the extracellular antioxidants, therefore the amount of H2O2 detected was greater than the exogenous H2O2 added to the sample. Because bacteria-treated cells...
produce higher levels of extracellular antioxidants, the
difference in antioxidant capacity between the two bacterial
strains, rather than untreated cells, is a better indication of
the magnitude of the oxidative burst (Fig. 1B). By 6 h the
oxidative burst reached the equivalent of 50 μM H2O2 per
sample.

Free H2O2 was detected in samples after 5 h by a
different chemiluminescent technique, which does not add
exogenous H2O2 (Fig. 1C). This coincided with the
approximate time where antioxidant capacity is zero
(Fig. 1A) confirming that the extracellular antioxidants
have been oxidized and any new production of extracellular
phenolics is not able to completely scavenge newly
produced H2O2.

3.2. Monitoring antioxidant capacity during
potato/bacterial interactions

Changes in the extracellular antioxidant capacity of
potato suspension cells treated with *P. syringae* pv. *syringae*
strains were generally similar to the results of tobacco
except that less antioxidant accumulated (Fig. 2). Because
the antioxidant capacity was lower, only 20 μM H2O2 was
added exogenously to potato samples. Untreated potato cells
started accumulating extracellular antioxidants immediately
and by 6 h produced levels sufficient to react with 10 μM
H2O2 (Fig. 2A). Cells treated with either strain of *P.
syringae* pv. *syringae* did not accumulate extracellular
antioxidants until 1–1.5 h after treatment. Comparison of
these samples with untreated cells suggested that a small
oxidative event had occurred, coincident with the addition
of the bacteria, and oxidized extracellular phenolics during
the first 2 h period preventing their accumulation (Fig. 2 A
and B). After about 2 h, cells treated with strain B7 (HR−)
increased in antioxidant capacity at a faster rate than
untreated cells and reached nearly 20 μM by 6 h. Cells
treated with strain WT (HR+) underwent a second
oxidative event equivalent to about 20 μM H2O2, after
5–6 h, when compared to B7 (HR−) treated cells.

Free H2O2 could be detected during the second oxidative
burst using the luminol dependent direct detection technique
without exogenous H2O2 (Fig. 2C). The H2O2 was detected
over the entire period of the oxidative burst, whereas in
tobacco, it was detected only after all the extracellular
phenolics had been oxidized. Two factors may contribute to
this earlier detection of free H2O2 in potato cells. Potato
cells have lower endogenous peroxidase activity (discussed
below) and lower concentrations of extracellular phenolics.
These two factors directly contribute to a lower on-going in-
situ rate of scavenging endogenous H2O2 by potato cells
compared to tobacco cells. This lower rate of scavenging
appears to increase the sensitivity of the H2O2 assay in
potato by allowing the exogenous luminol and peroxidase to
better compete for the endogenous H2O2.

3.3. Monitoring antioxidant capacity during
plant/heat-killed bacterial interactions

Fig. 3 shows the results of tobacco cells treated with heat-
killed strain WT (HR+) bacteria, which previous studies
[4] have shown elicits an oxidative burst. When heat-killed
bacteria were added to cells at either 0 or 4.5 h, an oxidative
burst could be detected. The first burst, 0 h, occurred
Immediately and peaked within an hour, after which the antioxidant capacity began to increase as extracellular antioxidants began to accumulate (Fig. 3A). The rate of accumulation was faster than untreated cells and by 4 h both untreated and treated cells had similar antioxidant capacities. Addition of heat-killed bacteria to untreated cells after 4.5 h also caused an immediate oxidative burst that peaked within an hour (Fig. 3A). Comparison of the 0 and 4.5 h treatments to untreated cells suggests that more H₂O₂ was produced by the 4.5 h response, 90 μM, than the burst at 0 h, 40 μM (Fig. 3B).

In contrast, direct detection of free H₂O₂ suggests that the oxidative burst at 0 h, 45 μM, was greater than the 4.5 h burst, 18 μM (Fig. 3C). This can be explained by the lower concentration of extracellular antioxidants at 0 h and therefore, the lower rates of scavenging during the first burst. The higher concentrations of extracellular phenolics at 4.5 h scavenge the H₂O₂ as it is being produced so that the concentration of free H₂O₂ remains lower. In addition as mentioned above, it appears that the luminol-dependent detection of H₂O₂ requires the luminol to compete with extracellular phenolics if present.

### 3.4. Parameters affecting the assay

The assay is based upon the peroxidase-dependent chemiluminescent reaction of luminol with H₂O₂. Fig. 4A shows a diagrammatic simplification of the enzyme reaction, which is complex and the subject of many mechanistic studies.[7,14,18] In reaction a, peroxidase (POX) is oxidized by H₂O₂ and loses two electrons. In each of the next two reactions, peroxidase gains back one electron from a donor substrate, which in this study would be either extracellular antioxidant or luminol. If the electron donor is luminol, light is generally produced from the unstable oxidized product (L*). We used a luminometer to measure the chemiluminescence, which corresponds to the near-steady state concentration of oxidized luminol, produced during the first 20 s of the reaction (Fig. 4B insert).

Under the conditions described here with H₂O₂ concentrations generally less than 100 μM and other assay reactants constant, the peak or maximum value of chemiluminescence is directly proportional to the H₂O₂ concentration (Fig. 4B). The extracellular antioxidant in suspension cells will reduce the chemiluminescence by scavenging of H₂O₂ (Fig. 4C). A final concentration of 50 μM H₂O₂ was added to tobacco suspensions that had been incubated for increased lengths of time and therefore contained increased amounts of antioxidant. The antioxidants, via endogenous peroxidase, reduce the hydrogen peroxide available for reaction with the luminol (Fig. 4C).

Slight variations in the concentration of luminol used in the present study, 170 μM, had minimal effect on the peak value of chemiluminescence under the current assay conditions (Fig. 4D).

The amount of chemiluminescence produced by this assay is also proportional to the peroxidase concentration as shown here with luminol, 170 μM, and H₂O₂, 10 μM, held constant (Fig. 4E). Because suspension cells contain endogenous cell wall peroxidase this must be considered for each cell line. Ideally the exogenous peroxidase should provide the majority of the enzyme for luminol oxidation to over shadow changes in endogenous peroxidase levels that may occur (Fig. 5). The traditional guaiacol-peroxidase reaction was adapted as described in the Section 2 to provide an estimation of the total extracellular peroxidase in cell suspensions including both soluble and cell wall bound enzyme. Using
Potato and tobacco suspension cells, it was apparent that peroxidase activity more than doubled over the 5–6 h monitoring period (Fig. 5). The increase in peroxidase was similar in untreated or bacteria-treated cells for both potato and tobacco. The final endogenous peroxidase levels for potato and tobacco cells accounted for 5 and 10%, respectively, of the total peroxidase activity utilized in the luminol-dependent assay. Bacterial pathogens at concentrations used in these experiments contributed less than 1% of the peroxidase activity. Therefore, when comparing various treatments, the slight changes in endogenous peroxidase should not greatly influence the results.

The production of phenolics and other antioxidants such as ascorbic acid in suspension cells can substantially affect the redox status of the extracellular region. The method described here provides a relatively simple and non-invasive means to quantify the extracellular antioxidants present in cell suspensions. In addition, by accounting for changes in the concentration of these antioxidants, this method can provide a better estimate of the timing and magnitude of oxidative bursts that may occur when cell suspensions are treated with pathogens or pathogen-related elicitors. The technique avoids the need to directly detect reactive oxygen species and accounts for scavenging that may have occurred. Variations of this concept can be used to detect oxidative events in other scenarios such as infection drops or intracellular fluid.
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References


