Phytochemistry, Vol. 21, No. 4, pp. 827–831, 1982.
Printed in Great Britain.

0031-9422/82/040827-05$03.00/0

QUANTIFICATION OF HYDROGEN PEROXIDE IN PLANT EXTRACTS BY THE CHEMILUMINESCENCE REACTION WITH LUMINOL

ERICH WARM* and GEORGE G. LATIES
Department of Biology and Molecular Biology Institute, University of California, Los Angeles, CA 90024, U.S.A.

(*Received* 18 *June* 1981)

Key Word Index—Hydrogen peroxide; chemiluminescence; plant tissues; ethylene; senescence; ripening.

Abstract—The chemiluminescence of luminol (3-aminophthalhydrazide) with H_2O_2 has been used to quantify endogenous amounts of H_2O_2 in plant tissues. The reaction is linear over at least three orders of magnitude between 10^{-5} and 10^{-2} M H_2O_2. Interference by coloured compounds in the crude extract is calibrated by a purification step with Dowex AG 1-X8. The extract is calibrated with an internal H_2O_2 standard, and the specificity verified by H_2O_2 purging with catalase. The minimum detectability for H_2O_2 of this assay is at least 1 ng, corresponding to 0.1–1 g fresh material. Data are presented for the levels of H_2O_2 in potatoes after treatment with oxygen and ethylene, in tomatoes before and after ripening and in untreated germinating castor beans as well as in beans treated with aminotriazol to inhibit catalase activity. Though data using the titanium test are generally confirmed, the method presented here has the advantage of higher sensitivity and specificity.

INTRODUCTION

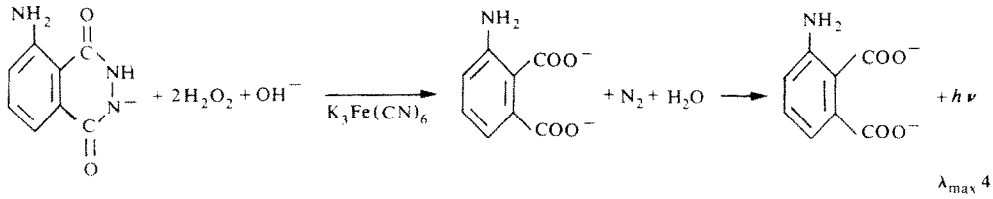
Whereas aerobic forms of life depend on oxygen as the ultimate electron acceptor in respiratory oxidative phosphorylation, pseudo-cyclic photophosphorylation also entails oxygen reduction with the superoxide anion radical and H_2O_2 as intermediates [1]. These intermediates may attack a variety of susceptible enzymes and ultrastructural cell components with damaging effects. While the oxygen radicals are short-lived in tissues, their product, H_2O_2, generated in large measure by superoxide dismutase, is a rather stable reduced oxygen form. The cell is well equipped with enzymes that degrade and consume H_2O_2 (catalase, peroxidases), thus protecting reduced cellular components against H_2O_2-mediated oxidative breakdown. At the same time, H_2O_2 plays an important role as an intermediate in several enzyme-catalysed reactions in both animal and plant cells. Its functional importance is established in several cases, e.g. in the bactericidal activity of polymorphonuclear leucocytes [2], and is discussed in the context of ageing and senescence of tissues and organs [8]. Our interest was focused on the role of H_2O_2 in the ageing of plant tissues and the ripening of fruit. It has been shown that the ripening of fruit, which is reflected by an upsurge of respiration and ethylene production [9, 10] is accompanied by an increase in H_2O_2 [3, 4]. Though the increases seem to occur generally in ripening fruit, the question persists whether H_2O_2 exerts a specific effect in the regulated events yielding the fruit climacteric, or whether it is merely a by-product of the changed metabolism. In analogy with the climacteric, storage organs treated with ethylene respond with a respiratory increase also associated with an accumulation of peroxides [5]. The high peroxidase activity in plant tissues casts some doubt on H_2O_2 measurements in crude extracts, and the conclusions drawn therefrom. Because of the possible central role of H_2O_2 and free radicals in cell life [6], we have developed a method for H_2O_2 quantification that takes into account its reactivity and degradability during the extraction procedure. In place of the titanium method [3], that is based upon the formation of an insoluble coloured complex between H_2O_2 and Ti^{2+}, we made use of the chemiluminescence (CL) attending the reaction of luminol (3-aminophthalhydrazide) with H_2O_2 for the quantification of H_2O_2 in crude extracts from fruit, seedlings and storage organs. Whereas this reaction has been described only for highly diluted and purified test samples [7, 8], we present a rapid test for H_2O_2 in extracts directly obtained from a wide variety of plant material, with sufficient proof of its H_2O_2 specificity. In conjunction with a chemiluminescence detector, as used for the firefly ATP assay, a very low detection limit can be achieved with good reproducibility and significance.

RESULTS AND DISCUSSION

Extraction procedure

The chemiluminescence attending the peroxidation of luminol by H_2O_2 is only possible when a catalyst and/or co-oxidant is added [7]. The catalyst/co-oxidant of our choice, ferricyanide, catalyses the oxidation of 6-aminophthalhydrazide to 6-aminophthalate with emission of N_2 and light according to Scheme 1.

*Present address: Botanisches Institut der Universitaet, Menzingerstr. 67, D-8000 Muenchen 19, West Germany.



Scheme 1. Ferricyanide catalysed peroxidation of luminol by H_2O_2.

The emission response of this reaction is highly pH-dependent, with an optimum at *ca* pH 11. However, there is increasing chemiluminescence with air under alkaline conditions, making pH 9.5 advisable as a compromise between a sufficient quantum yield and a low background in the blank. The pH dependency in addition necessitates a buffer system with sufficient capacity to overcome the acid content of the added extracts that usually are *ca* pH 5. Preliminary tests showed that 0.2 M ammonium hydroxide, pH 9.5, maintained the pH without changing the CL response. The time-course of light emission from the onset of CL shows a sharp decrease in accordance with published kinetics [7, 8]. Nevertheless, highly reproducible readings were obtained by integrating light pulses over a preset time, usually 5 sec, immediately following the start of the reaction. As shown in the calibration curve (Fig. 1), integration of the signals for 5 sec resulted in 10^5–10^8 net impulses for a concentration range of 20 μM–10 mM H_2O_2. This indicates a detection limit of 1 nmol H_2O_2. The extraction procedure had to take into account the reactivity of H_2O_2 and the activity of degrading enzymes in the tissues. Among the several extraction methods applicable, the best results were obtained by homogenizing quick-frozen material in 5% TCA (see Experimental). The complete inhibition of H_2O_2-degrading activity by this method was verified by the following test. Potato tissue (5 g) was extracted in 20 ml water to permit digestion of endogenous H_2O_2 by catalase present in the homogenate and the extract clarified by centrifugation. (A) 100 μl of the clear 12 000 *g* supernatant (water-extract) was added to 2.0 ml ice-cold 5% TCA containing 25 nmol H_2O_2. This was to test for inactivation of potato catalase by TCA. (B) 25 μl catalase suspension (4 mg/ml in 50 mM Tris–HCl, pH 7.0) was added to mixture (A). (C) 100 μl water-extract and 25 μl catalase supension were incubated for 5 min at room temperature together with 1.8 ml water containing 25 nmol H_2O_2. This test served both as a control for catalase activity and as a test for specificity of the method. The reaction was terminated with 0.2 ml 50% TCA. The samples were further processed and peroxide determined as described in the Experimental. The results (Table 1) showed that H_2O_2 was degraded neither by the extract nor by catalase, when 5% TCA was used as a denaturing reagent.

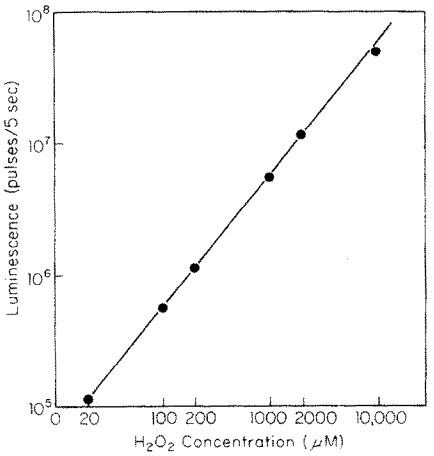


Fig. 1. Calibration curve for the chemiluminescence of luminol with H_2O_2 in water. Light pulses were integrated for 5 sec.

To further ensure that during extraction and subsequent treatment any loss of H_2O_2 is detected and compensated for, an aliquot of the material to be tested was internally standarized by adding a given amount of H_2O_2, usually 3 μmol, directly to the extraction medium before homogenization of the material. Reference standards consisted of the extraction medium (5% TCA) plus the same amount of H_2O_2 as added to the extracts. Depending on the plant material, we found that the recovery of the

Table 1. Test for the inactivation of H_2O_2-degrading enzymes by 5% TCA, the extraction medium for H_2O_2

Test condition*	Chemiluminescence		
	Total (pulses/5 sec)	Deviation (σ, %)	Relative rate (%)
(A) Extract + H_2O_2 + TCA	1 129 400	2.2	100
(B) Extract + H_2O_2 + catalase + TCA	1 095 600	1.8	97
(C) Extract + H_2O_2 + catalase − TCA	15 169	3.8	1.3

*See text for details.

added standard was sometimes drastically diminished despite that fact that the degrading enzymes were inactivated. In these cases, the extracts were more or less yellow, and we assumed that the coloured compounds interfered with the CL by quenching the emitted light rather than by directly affecting the amount of H_2O_2. After removing the coloured components (absorbance in the range 400–450 nm was less than 0.05), recovery was up to 95%. Though luminol CL should be specific for H_2O_2, there may be some doubt whether the CL due to the extract derives solely from H_2O_2 or from other peroxides as well. To test this point, 500 units of catalase were added to an aliquot of the purified extract and the CL recorded again after incubation. The amount of H_2O_2 was thus calculated from the difference in chemiluminescence before and after addition of catalase (ΔCL). The background luminescence varied in the extracts, but accounted for only 10% at most of the H_2O_2 (catalase-digestible) CL. Tomato fruits were an exception, with significantly higher residual, or background, CL, as pointed out later. The nature and cause of this CL is not clear, but may be caused by other peroxides in the crude extracts.

Measurement of H_2O_2 in potatoes

Potato tubers treated with ethylene show an upsurge in respiration as well as an increase in endogenous peroxides, both of which are enhanced when ethylene is presented together with pure oxygen (Fig. 2). The peroxide levels, as determined by the titanium method [3], are low at best, and this condition, together with the abundance of extraneous components in potato extracts, makes the latter a useful material to test the chemiluminescence method. Tubers were treated with 10 μl/l. ethylene in oxygen for 24 hr and H_2O_2 levels in treated tubers were compared with H_2O_2 concentrations in tubers held in oxygen alone. The result as presented in Table 2 shows an increase in H_2O_2 of 41% in the ethylene treated tubers. The residual CL was negligible in both groups.

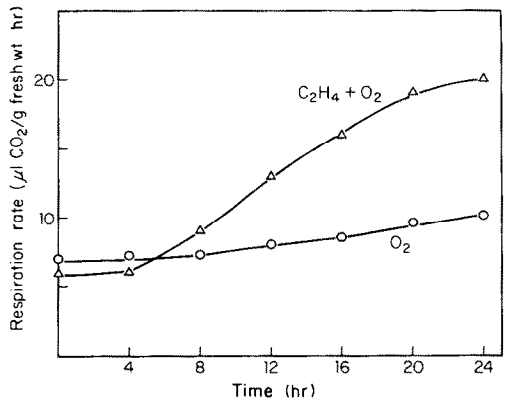


Fig. 2. Effect of ethylene in oxygen on the respiration of potato tubers. Ethylene, 10 μl/l.

Measurement of H_2O_2 in tomatoes

We used tomatoes for the quantification of H_2O_2 because they were easily available in the green unripe and the red ripe stages and the degree of ripening could be measured by the rate of evolution of ethy-

Table 2. H_2O_2 in potato tubers treated with oxygen (control) or oxygen plus 10 μl/l. ethylene (ethylene). 3 μmol H_2O_2 added to extracts as internal standard. ΔCL represents the difference in chemiluminescence before and after addition of catalase to aliquots of sample. Net CL, standard, obtained by subtracting the CL-sample from the CL of sample plus internal standard. CL-sample is corrected by dividing ΔCL-sample by the % recovery of standard H_2O_2 added to samples. For further explanation see text and Experimental

	Control	Ethylene	Control + standard H_2O_2	Ethylene + standard H_2O_2	Standard H_2O_2
ΔCL (pulses/sec)	134 640	190 060	494 900	541 090	415 590
τ% (ΔCL)	0.8	1.9	1.2	1.9	1.3
Net ΔCL, standard			360 260	361 030	415 590
% Recovery, standard			86.7	86.9	100
CL-sample, corrected	155 294	218 711			
H_2O_2, nmol	1120	1579			3000
H_2O_2, μmol/kg fr.wt	224.0	315.8			

Table 3. H_2O_2 in green unripe tomatoes and ripe red tomatoes. Details as in Table 2

	Green tomatoes	Red tomatoes	Green tomatoes + standard H_2O_2	Red tomatoes + standard H_2O_2	Standard H_2O_2
ΔCL (pulses/5 sec)	55 000	58 200	414 600	403 800	521 000
σ% (ΔCL)	4.1	5.1	5.9	6.2	7.7
Net ΔCL, standard			359 600	345 600	521 000
% Recovery, standard			69.0	66.3	100
CL-sample, corrected	79 800	87 800			
H_2O_2, nmol	460	510			3000
H_2O_2, μmol/kg fr.wt	92	102			

Table 4. H_2O_2 in germinating castor beans in water (control) or aminotriazol. Details as in Table 2

	Control	Aminotriazol	Control + standard H_2O_2	Aminotriazol + standard H_2O_2	Standard H_2O_2
ΔCL (pulses/5 sec)	116 800	293 700	625 800	788 600	532 800
σ% (ΔCL)	1.7	1.6	1.1	1.1	1.9
Net ΔCL, standard			509 000	494 900	532 800
% Recovery, standard			95.5	92.8	100
CL-sample, corrected	122 300	316 200			
H_2O_2, nmoles	690	1780			3000
H_2O_2, μmoles/kg fr.wt	138	356			

lene. While the amount of ethylene produced from green tomatoes was as low as 0.1 μl/kg/hr, the rate in red fruits reached 25 μl/kg/hr. The H_2O_2 level, however, showed an increase of only 10% in red fruit compared with green (Table 3). Frenkel and Eskin [4] claimed much higher values. Red tomatoes, however, gave a residual CL after catalase incubation of the same magnitude as in its absence. In other words, ripe tomatoes contain water extractable (5% TCA) compounds not degraded by catalase that cause CL and which may account for the discrepancy.

Measurements of H_2O_2 in castor beans

We wanted to extend our investigation to a system where the amount of H_2O_2 can supposedly be changed by affecting the activity of endogenous catalase. The germinating castor bean is known for its high amount of catalase, the enzyme degrading H_2O_2 in the cell [11]. Inhibition of this enzyme should cause H_2O_2 accumulation in the tissue. 3-Amino-1, 2, 4-triazole is known for its specific effect in depressing catalase activity in animal and plant cells [12, 13]. Castor beans were imbibed for 6 days with 1 mg/ml aminotriazole (controls with water) and the activity of catalase (H_2O_2:H_2O oxidoreductase, EC 1.11.1.6) tested as an indicator of the inhibitory effect of the drug. Further tests for endogenous H_2O_2 were carried out as described earlier. The inhibition of catalase activity, as measured by H_2O_2 production *in vivo*, is more than 93%, with values of 50 μmol and 810 μmol H_2O_2/min/mg protein in the treated and untreated, castor beans respectively. This decreased catalase activity is reflected by an almost threefold increase in H_2O_2, as shown in Table 4.

Summarizing, the application of the chemiluminescence method to quantify endogenous levels of H_2O_2 gave specific and reproducible data with various plant materials. Internal standardization seems to be a reasonable way to overcome interference by cellular components in crude extracts. Whereas increases of H_2O_2 undoubtedly occur with ripening and ethylene-mediated stimulation of respiration, its magnitude varies largely and accordingly provides no clue as to whether H_2O_2 plays a physiological role in these developmentally controlled processes.

EXPERIMENTAL

Plant material. Potato tubers (*Solanum tuberosum* L.) were obtained from local markets; tomato fruits (*Lycopersicon esculentum* Mill.) harvested in the green and red stages of ripening were the kind gift of Professor Harry Highkin; castor bean seeds (*Rhizinus communis*) were obtained from a commercial source.

Measurement of respiration. Potato tubers were treated with either O_2 or O_2 and 10 μl/l. C_2H_4 for 24 hr and the respiration recorded automatically using an IR absorbance analyser (Anarad AR 500). Following treatment, central cylinders of potato tubers were removed with a 1-inch cork borer and immediately sliced into liquid N_2.

Measurement of C_2H_4 production. Tomatoes at the green and red stages of ripening (*ca* 250 g each) were kept in a closed glass jar for 4 hr and gas samples of 20 ml were withdrawn from the head space, and the concn of C_2H_4 determined with an F & M Gas Chromatograph calibrated with a C_2H_4 standard. The whole fruits were immediately sliced into liquid N_2.

Germination of castor beans. The outer coats of the seeds were removed, and the seeds imbibed in a Petri dish either in dist. H_2O or in 1 mg/ml 3-amino-1, 2, 4-triazole (gift from Dr. D. Appleman) and incubated at 30° and 80% humidity for 6 days. Samples from both groups were taken for catalase assay and H_2O_2 measurements.

Measurement of catalase activity. 2 g castor beans incubated as noted above were homogenized for 15 sec with a Polytron homogenizer in 5 ml 0.04 M Tris–HCl buffer, pH 7, containing 0.5% (w/v) Polycar AT. The homogenate was passed through Miracloth (Chicopee Mills, Milltown, NJ) and centrifugated for 20 min at 12 000 *g*. The activity in the extract was measured by O_2-release from H_2O_2 as determined with a Clark type oxygen electrode. 2 ml buffer (0.02 M Tris–HCl, pH7) were deoxygenated with N_2 in a vessel at 25°, and 20 μl M H_2O_2 and 5 μl Triton X-100 (2%) were added. After injecting 25 μl extract, the O_2 evolution was recorded for 10 min.

Measurement of H_2O_2. The extraction of H_2O_2 was generally the same for all species. 5 g of the frozen sliced material was transferred into 20 ml ice-cold 5% TCA and homogenized in a Virtis blender for 2 min. For internal standardization between 1 and 5 μmol H_2O_2 was added to a parallel sample prior to the homogenization, and to a blank of 25 ml 5% TCA without plant material. The crude extracts were centrifuged for 30 min at 12 000 *g*. 1 ml aliquots of the supernatants were passed twice over a 0.7 × 4 cm column of 100 mg Dowex anion exchange resin (AG 1 × 8, Bio-Rad). This step removed most of the coloured components in the extracts. The blank and standard were treated the same way. The amount of H_2O_2 in the resulting extract and in the standards was tested by pipetting 50 μl test soln into 50 μl 0.5 mM luminol (3-aminophthalhydrazide, Aldrich) in 0.2 M NH_4OH (pH 9.5) in 0.9 ml test tubes. The test tube was placed in the measuring cell of an Aminco Chem Glow Photometer and chemiluminescence (CL) initiated by injecting 100 μl 0.5 mM $K_3Fe(CN)_6$ (in 0.2 M NH_4OH) into the

mixture. The emitted photons were counted over 5 sec with a pulse integrator (Varian Aerograph Mod 485). The specificity of the CL-reaction for H_2O_2 was verified by adding 500 units of catalase (bovine liver, Calbiochem) to 1 ml of the decolorized extract (buffered with Tris–HCl pH 7). After incubation for 10 min at 30° the CL (designated as residual CL) was recorded again. The difference between the two measurements is presented as H_2O_2-specific CL (ΔCL). For compensation of any degradation of H_2O_2 and/or interference by other components, a fixed amount of H_2O_2 was added to the aliquot as int. standard. The difference in CL between the extract and the extract plus standard H_2O_2 was expressed as net CL, and the recovery of added peroxide in relation to the standard (without plant material) was calc. The net CL was then corr. according to the % recovery, which varied depending on the extract.

Acknowledgements—This work was supported by an award of the Deutsche Forschungsgemeinschaft to E. W. and a grant from the U.S. Public Health Service to G. G. L.

REFERENCES

1. Elstner, E., Stoffer, C. and Heupel, A. (1975) *Z. Naturforsch. Teil C.* **30**, 53.
2. Klebanoff, S. J. (1967) *J. Exp. Med.* **126**, 1063.
3. Brennan, T. and Frenkel, C. (1977) *Plant Physiol.* **59**, 411.
4. Frenkel, C. and Eskin, M. (1977) *Hortic. Sci.* **12**, 552.
5. Chin, C.-K. and Frenkel, C. (1977) *Plant Physiol.* **59**, 515.
6. Harman, D. (1962) *Radiat. Res.* **16**, 753.
7. Roswell, D. F. and White, E. H. (1978) *Methods in Enzymology* Vol. 57, pp. 409–424.
8. Seitz, W. R. (1978) *Methods in Enzymology* Vol. **57**, pp. 445–462.
9. Burg, S. P. and Burg, E. A. (1962) *Plant Physiol.* **37**, 179.
10. Abeles, F. B. (1973) *Ethylene in Plant Biology*, pp. 167–176. Academic Press, New York.
11. Huang, A. H. C. and Beevers, H. (1971) *Plant Physiol.* **48**, 637.
12. Pyfrom, H. T., Appelman, D. and Heim, W. G. (1957) *Plant Physiol.* **32**, 674.
13. Racusen, D. (1958) *Arch. Biochem. Biophys.* **74**, 106.