Plant Growth Regulation (2006) 48:89–95
DOI 10.1007/s10725-005-5089-y

An improved chemiluminescence method for hydrogen peroxide determination in plant tissues

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Received 28 March 2005; accepted in revised form 13 November 2005

Key words: Grapevines, Hydrogen peroxide, Luminol chemiluminescence

Abstract

As a consequence of the increasing importance of hydrogen peroxide in plant metabolism, more efficient methods are required for accurate determinations of its concentration in plant tissue and organs. Here we present a highly sensitive chemiluminescence (CL) method based on the Co (II) catalysed oxidation of luminol by H_2O_2. The replacement of ferricyanide, the traditional catalyst of luminol luminescence by Co (II), enhanced the sensitivity of the reaction towards H_2O_2 in three orders of magnitude. Thus, plant extracts can be diluted to such a level that quenching effects of phenols and ascorbic acid (ASA), which are normally present at high concentrations in plant tissues is avoided, and therefore, pre-treatments with PVP and ascorbate oxidase to remove these quenchers from plant-extracts become unnecessary. To exemplified the high performance of the method, measurements of H_2O_2 were carried out in PVP treated and non-treated extracts of grapevine leaf, a plant tissue that contain high levels of phenols and ASA. Moreover, increases in H_2O_2 levels were detected in disc-leaf treated with aminotriazole, a specific Cat inhibitor, showing the importance of Cat as a H_2O_2 scavenging enzyme in leaves of grapevine.

Introduction

In addition to its role in oxidative damage, hydrogen peroxide (H_2O_2) is emerging as a key signalling molecule generated by plants in response to both biotic and abiotic stresses such as drought, UV-radiation, ozone, high and low temperatures and pathogen attacks (Prasad et al. 1994; Bartosz, 1997; Foyer et al. 1997; Dat et al. 2000). In green tissues, H_2O_2 is produced at high flux rate in chloroplast (Asada 1999) and in mitochondria (Moller 2001) via electron transport. In chloroplast, reduced ferredoxin is a strong reductant that provides electron to a variety of reaction, including the reduction of oxygen to superoxide which in turn is rapidly disproportionate to H_2O_2 in a catalysed or spontaneous reaction (Chitnis 2001). In mitochondria, the ubiquinone pool is a major site of superoxide production and therefore of H_2O_2 (Moller 2001). In addition there are a number of other enzymes in plant tissues that are capable of producing significant amounts of H_2O_2, including peroxidases, NADPH oxidases and oxalate oxidases (Levine et al. 1994; Berna and Bernier 1999; Bolwell 1999). H_2O_2 is moderately reactive and is a relatively long-lived molecule that can diffuse across membranes and inactivate enzymes by oxidizing thiol groups (Takeda et al. 1995),

therefore their accumulation must be under tight control. Plant possesses very efficient enzymatic and non-enzymatic antioxidant defence systems that allow scavenging of H_2O_2 and protect plant cells from oxidative damage (Asada 1999).

As a consequence of the increasing importance of H_2O_2 in plant metabolism, adequate and efficient methods are more necessary to determine accurately its concentration in plant tissue and organs. Although the existence of a great number of analytical methods for H_2O_2 determinations such as the fluorometric (Genfa and Dasgupta 1992), spectrophotometric (Ngo and Lenhof, 1980) and chemiluminescent (Warm and Laties 1982), measurements of H_2O_2 concentration shows great variability in the literature. Differences in the application of existing methods appear to yield very variable results ranging in most cases between 0.1 and 1 μmol g^{-1} fr.wt. Recently Veljovic-Jovanovic et al. (2002), have claimed that such values are too high, since key metabolic reactions, including CO_2 fixation and tricarboxylic acid cycle are inhibited by as little as 10 μM H_2O_2.

Here, we present an improvement to the chemiluminescent method developed by Warm and Laties (1982) based on the luminescence of luminol. The replacement of ferricyanide by Co (II) as catalyst for the oxidation of luminol by H_2O_2 improved the sensitivity of the reaction towards H_2O_2 in three orders of magnitude. Thus, with the increasing sensitivity towards H_2O_2, plant-extracts can be diluted to such a level that interference to chemiluminescence (CL) by phenolics and ascorbate, which are normally present at high concentrations in plant tissue, is avoided. Therefore, pre-treatments of plant-extracts with insoluble PVP and ascorbate oxidase prior to CL measurements become unnecessary.

Material and methods

Plant materials

Plants of *Vitis vinifera* L. cv. Thompson seedless were obtained from a plantation in the central Valley of Chile. The 4×4 m planted vines were eight years old, drip irrigated and grown under a horizontal system. Experiments were carried out on leaf-discs.

Reagents

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), cobalt (II) chloride and sodium carbonate were purchased from Aldrich (U.S.A.). H_2O_2 30% w/v standardised by the Kingzett's method Treadwell and Hall (1937), was purchased from Merck, (Germany) and insoluble polyvinylpolypirrolidone (PVP) was obtained from Sigma Chemical (U.S.A.). All other chemicals were of reagent grade and used without further purification. Stock luminol solution was prepared by dissolving 11.5 mg of luminol in 10 ml of sodium carbonate buffer solution pH 10.2. Stock Co (II) solution was prepared by dissolving 7.14 mg Co (II) chloride in 10 ml carbonate buffer. Stock mixed reagent solution was prepared by diluting 10 ml stock luminol and 2 ml stock Co (II) solutions in 100 ml 0.1 M sodium carbonate pH 10.2. The freshly prepared mixed solution was stored in the dark for at least one hour and then diluted 10 fold. The diluted mixed reagent solution was stored in the dark for 12 h before used. Although the non-diluted mixed reagent solution is usable its sensitivity was lower.

Determination of H_2O_2 content by the Co (II) catalysed oxidation of luminol

Grapevines leaves (0.1 g) were ground to fine powder in liquid N_2 then extracted with 0.5 ml of 5% trichloroacetic acid (TCA). The homogenate was centrifuged for 10 min at 13,000*g*. Insoluble PVP 5% (w/v) was added to the homogenate in some samples. Samples were diluted with carbonate buffer and a 20 μl aliquot of the diluted sample was incubated for 15 min at 30 °C with 5 μl (50 U) catalase (bovine liver, Sigma, USA) or with 5 μl of distilled water. After incubation, luminescence was measured by adding 2 μl of the catalase treated or non-treated samples to 1 ml of the diluted mixed reagent solution. The emitted photons were counted over 5 s. with a luminometer HY-LITE® 2 (Merck, Germany). The difference between catalase treated and

non-treated samples (ΔCL) was considered as H_2O_2 specific CL.

Enzymes activities

Leaf discs were ground to fine powder in liquid N_2. The powder extracted with buffer (0.5 M Tris–HCL, pH 7.5, 5 mM DL-dithiothreitol (DTT), 1 mM $MgCL_2$, 10 μM phenylmethanesulfonyl fluoride (PMSF), 2% insoluble PVP, 2% Triton X-100 and 12.5% glycerol), centrifuged for 15 min at 13,000 g. Catalase activity was determined in the supernatant following O_2 evolution using a Clark-type oxygen electrode (Hansatech, UK). Measurements were carried out at 30 °C in 1 ml 0.1 M phosphate buffer pH 7.0 in the presence of 1 mM H_2O_2. Ascorbate peroxidase was determined by the method described by Amako et al. 1994.

Effect of aminotriazole and hydroxyurea on Catalase activity and H_2O_2 levels

Leaf discs, 2.7 cm in diameter were incubated overnight with continuous irradiance (200 μmol m^{-2} s^{-1}) in solutions con10 mM 3-amino-1,2,4-triazole or with 10 mM hydroxyurea. Control discs were incubated under the same conditions in distilled water. Three discs per treatment were used for H_2O_2 determinations and other three for catalase and ascorbate peroxidase (APx) activities and proteins.

Protein determination

Protein concentration was determined by the Bradford method (Bradford 1976).

Results and discussion

Several analytical methods have been developed for hydrogen peroxide determinations in plant tissues. The luminol CL method developed by Warm and Laties (1982) based on the ferricyanide catalysed oxidation of luminol by H_2O_2 is widely used. However, due to the high concentration of phenols and ascorbic acid (ASA) in plant extracts that quench luminescence, addition of PVP and ascorbate oxidase are required in order to remove phenols and ASA from extracts (Warm and Laties 1982; Veljovic-Jovanovic et al. 2002). Since Co (II) is the most sensitive luminol metal catalyst (Yuan and Shiller 1999), the sensitivity of the reaction toward H_2O_2 can be improved in three orders of magnitude if ferricyanide is replaced by Co (II) as catalyst. Figure 1 shows the CL of luminol in the presence of different concentrations of H_2O_2 using either ferricyanide or Co (II) as catalyst. Figure 2 shows quenching effect of ASA and ferulic acid on the Co (II) catalysed reaction of luminol in the presence of different H_2O_2 concentrations. In both cases, the quenching effect became less significant at concentrations lower than 0.1 μM.

Measurements of H_2O_2 in leaves of grapevine

Because leaves of grapevines contains high amount of phenols and flavonoids, 0.1–0.2 mmol per gram of fr.wt. (Kolb et al. 2001), which is about 10 fold the concentration of ASA (Pérez et al. 2002) their extracts were used for studying the effect of PVP and dilution on the determination of H_2O_2 by the Co (II) catalysed luminescence of luminol. To assure that the luminol CL be specific for H_2O_2, one fraction of the extract was treated with catalase (Cat) and the difference in CL between Cat non-treated and Cat treated fractions (ΔCL) was considered as CL due specifically to H_2O_2. To determine the required amount of PVP necessary to remove phenols from grapevine leaf extracts, different percentage of PVP (w/v) were added to the extracts and measurements of ΔCL were carried out. Figure 3 shows that ΔCL increased linearly with increases in the amount of added PVP, however, over 5 % ΔCL reached nearly a plateau, indicating that beyond that PVP concentration phenols are completely removed from the extract. Table 1, show the effect of dilution on ΔCL and in H_2O_2 content in PVP treated and non-treated extracts. At low levels of dilution, ΔCL values were lower in non-treated than in PVP treated extracts. Low ΔCL values observed in non-treated extracts were due to quenching effect of phenols, however, with increases in dilution, differences in ΔCL decreased

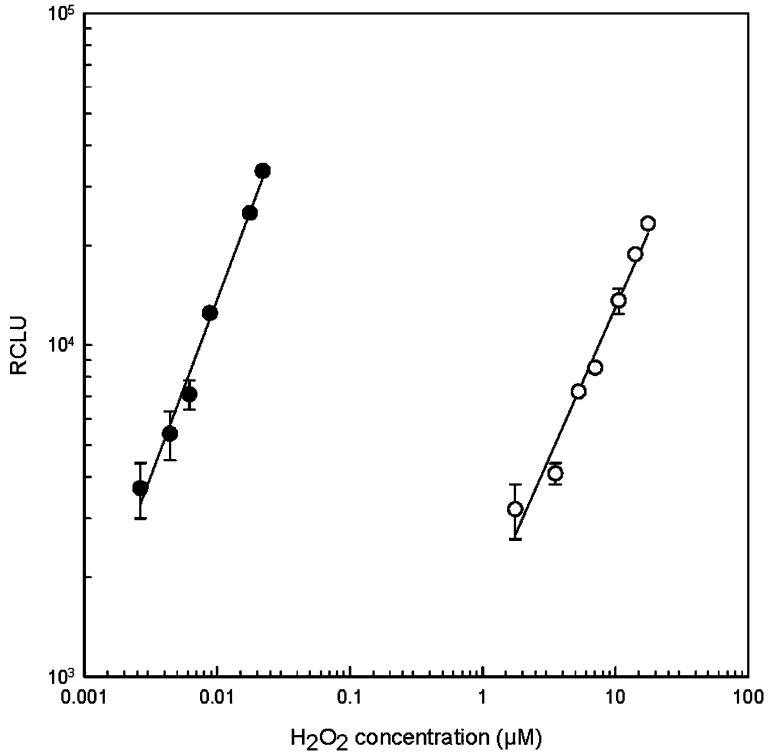

Figure 1. Determination of H_2O_2 by the chemiluminescence of luminol catalysed either by ferricyanide (○) or Co (II) (●). Values for H_2O_2 concentration are placed in logarithmic scale to remark the differences between catalyst. RCLU (relative chemiluminescence units).

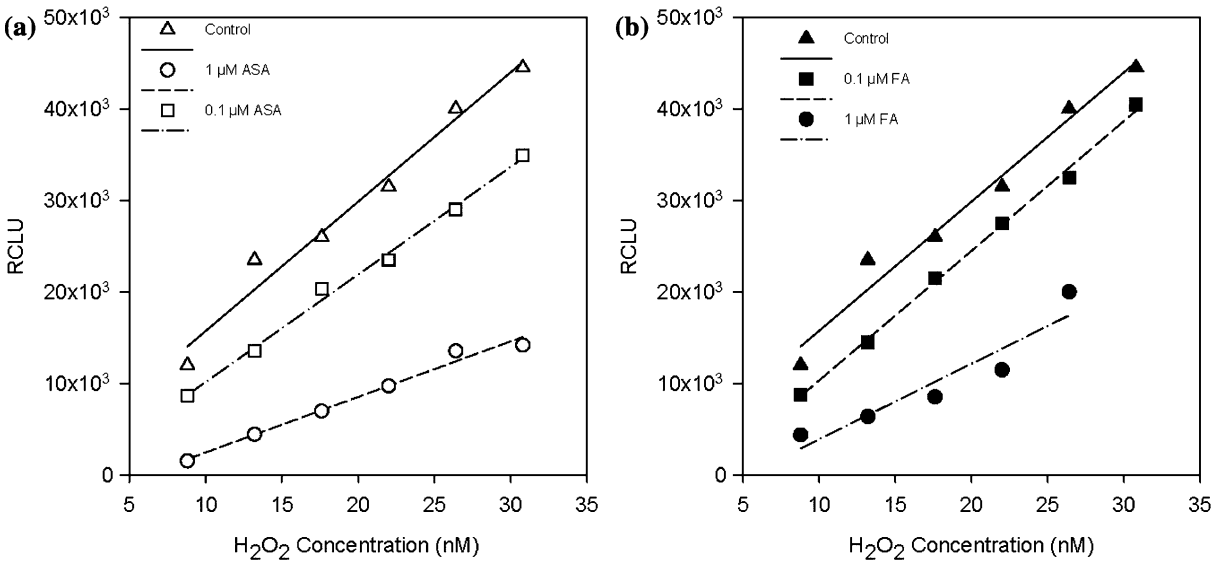

Figure 2. Quenching effect of ascorbic ASA (a) and ferulic acid (b) on the chemiluminescence of luminol catalysed by Co (II) in the presence of different concentration of H_2O_2. The concentration of quencher was 1 (O) and 0.1 μM (□). RCLU (relative chemiluminescence units).

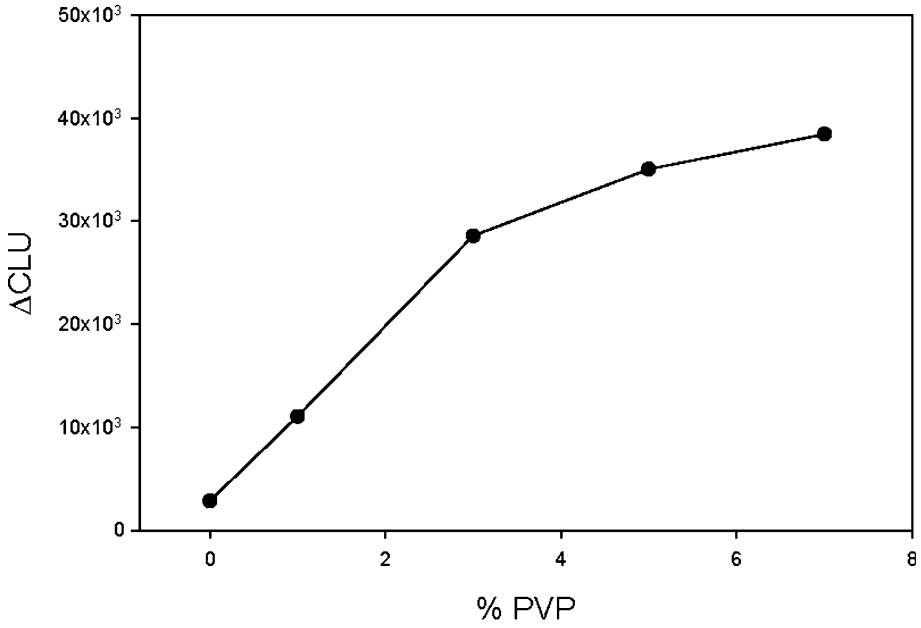

Figure 3. Effect of added PVP (w/v) on *Δ*CL in grapevine leaf extracts. *Δ*CL, correspond to differences in RCLU between Cat non-treated and Cat treated extracts. Measurements were carried-out by the Co (II) catalysed reaction of luminol.

Table 1. Determination of H_2O_2 content by the Co(II) catalysed reaction of luminol in PVP treated and non-treated extracts of grapevine leaf.

Dilution factor	Non PVP treated extracts		PVP treated extracts	
	*Δ*CL (RCLU)	H_2O_2 (nmol $\times g^{-1}$ fr.wt.)	*Δ*CL (RCLU)	H_2O_2 (nmol $\times g^{-1}$ fr.wt.)
100	7500	1.94 ± 0.3	74000	14.5 ± 3.1
250	10600	6.30 ± 1.1	41000	20.6 ± 4.5
500	14300	16.1 ± 3.2	23900	25.1 ± 5.3
1000	11400	26.7 ± 4.9	10100	24.3 ± 4.8

Extracts were diluted as indicated in 0.1 M buffer carbonate pH 10.2. Values are average of three independent replicates with the respective s.d.

and at a 1000 fold dilution disappeared completely between PVP treated and non-treated extracts, indicating that quenching effect of phenols became negligible at that level of extract dilution. Although PVP is very efficient in removing phenols and flavonoids it does not remove ASA. Thus, the estimated content of H_2O_2 in PVP treated extracts in which phenols have been removed, was lower in the low dilution level than in the highly diluted extracts (Table 1). This fact was due to the quenching effect of ASA that was not removed by PVP treatments. However, after diluting the PVP-treated extract over 100 fold, estimated values for H_2O_2 content were similar regardless of the dilution considered (Table 1). Therefore, reliable results for H_2O_2 determinations in grapevine leaves by the Co (II) catalysed oxidation of luminol, can be obtained by diluting PVP treated extracts over 100 fold or diluting non-treated extracts 1000 fold. The high sensitivity of the Co (II) catalysed reaction of luminol, with a nanomolar detection limit for H_2O_2, make possible to dilute plant extracts to such a level that quenching effect of ASA and phenols become negligible, and therefore pre-treatment of extracts with PVP and ascorbate oxidase become unnecessary.

Table 2. Effect of aminotriazole (AT) and hydroxyurea on Cat activity and on H_2O_2 levels in leaves of grapevines.

Treatment	Proteins (mg×g^{-1} fr.wt)	Cat activity (U×mg^{-1} Prot)	H_2O_2 (nmol ×g^{-1} fr.wt)
Control	2.89 ± 0.98	0.11 ± 0.03	24.1 ± 4.5
AT	1.86 ± 0.76	0.03 ± 0.006	43.8 ± 3.4
Hyroxyurea	2.63 ± 0.67	0.12 ± 0.05	26.1 ± 2.4

Leaf discs were incubated overnight in 10 mM AT and hydroxyurea solutions with continuous irradiance of 200 μmol m^{-2} s^{-1}. Controls were carried-out in distilled water under the same conditions. Values correspond to the average of three independent replicates.

Effect of aminotriazole and hydroxyurea on level of H_2O_2 in grape leaves

Catalase (Cat) and ascorbate peroxidase (APx) are the main H_2O_2 scavenging enzymes present in plant tissues (Asada 1999). Their inhibition by aminotriazole and hydroxyurea, specific inhibitors of Cat (Prasad 1997) and APx (Chen and Asada 1989) should increase H_2O_2 levels in plant tissues. Table 2, show the importance of Cat as a H_2O_2 scavenging enzyme in leaves of grapevine, since aminotriazole upon inhibiting its activity in 73% increased the levels of H_2O_2 in 83% in relation to control leaves, whereas, hydroxyurea did not altered H_2O_2 levels in leaf discs of grapevines.

Acknowledgements

The financial support of FONDECYT project 1050285 is gratefully acknowledged.

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