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A modified chemiluminescence method for hydrogen peroxide determination in apple fruit tissues☆

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ABSTRACT

Hydrogen peroxide (H_2O_2) is one of the important by-products produced by plant and fruit tissues during normal metabolism as well as under stress conditions. Evidence suggests that it is actively involved in many physiological activities in plants, including ripening, senescence and the development of disorders. Quantitative measurement of H_2O_2 in fruit has been a challenge due to variations in methodologies, and their sensitivities and interferences present in plant samples. Among the currently used methodologies, chemiluminescence (CL) is one of the most promising, due to its high specificity and sensitivity. However, direct application of CL methods developed for leaf analysis is not suitable for fruit, especially fruit peel tissues, possibly due to interfering compounds in fruit tissues. In this study, evaluation of the efficiency of removal of interfering compounds by PVP, PVPP and activated charcoal revealed that the PVPP is the most effective compound to remove the interference. This modified protocol can measure H_2O_2 content in apple peel and flesh tissues. 'Red Delicious' apple peel and flesh tissues were measured with amount of 1.48 and 1.03 μmol/g FW, respectively. The established protocol can also be used for a wide variety of tissues in addition to apple fruit, including strawberry tissues (fruit, calyx and leaves) and spinach leaves. This protocol was applied to determine the H_2O_2 concentration in 1-MCP and DPA treated apples after 5 months of storage, but no significant difference in H_2O_2 in those samples was found. Direct comparison of CL with a commercial hydrogen peroxide measurement OXIS kit was also made. The challenges to accurately assay H_2O_2 in fruit/plant tissue were discussed.

1. Introduction

Hydrogen peroxide (H_2O_2) is one of the reactive oxygen compounds (ROS) generated as a by-product in plant tissues during normal metabolism as well as under different stress conditions, such as oxidative stress, anoxia, pathogen attacks, extreme temperatures, drought, ozone, wounding and senescence (Blokhina et al., 2003). Mechanisms of H_2O_2 generation in plant biological systems and its physiological role as a key signalling molecule in environmental stress response and in controlling plant programmed cell death have been investigated (Foyer et al., 1997; Bolwell, 1999; Dat et al., 2000; Neill et al., 2002; Gechev and Hille, 2005). Reactive oxygen species and H_2O_2 are also believed to be important in fruit metabolism. In many fruits, reactive oxygen species including H_2O_2 are reported to be associated with fruit development, ripening and senescence (Brennan and Frenkel, 1977; Jimenez et al., 2002; Woods et al., 2005; Thompson, 1985); physiological disorders such as apple superficial scald (Rao et al., 1998); internal browning of pear flesh (Pinto et al., 2001) and anthocyanin degradation of blueberry fruit (Kader et al., 2002). It is necessary to determine the concentration of H_2O_2 in different stages of growth in the plant and fruit tissues to reveal its biological role.

Numerous studies report techniques for quantitative determination of H_2O_2 in plant tissues, especially for leaves. Two major colorimetric assays include one based on the color complex formed between titanium and the H_2O_2 (Brennan and Frenkel, 1977), and a second based on the oxidation of ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}) by H_2O_2 under acid conditions (Gay et al., 1999). In the latter, the ferric ions bind with the indicator dye xylenol orange to form a stable color complex, which can be measured at 560 nm. In addition, commercial measuring kits, such as the Bioxytech H_2O_2-560 colorimetric assay (OXIS International, Portland, Ore) are available. Both assays have been widely applied on plants tissues (Queval et al., 2008). Other spectrophyotometric techniques have also been developed (Jana and Choudhuri, 1981; Patterson et al.,

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Abbreviations: 1-MCP, 1-methylcyclopropene; CA, controlled atmosphere; DPA, diphenylamine; PVP, polyvinylpyrrolidone; PVPP, polyvinylpolypyrrolidone; AC, activated charcoal; Luminol, 5-amino-2,3-dihydro-1,4-phthalazinedione.

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1984; Li et al., 2000; Zhou et al., 2006). Fluorescence (Jimenez et al., 2002) and chemiluminesce (Warm and Laties, 1982; Perez and Rubio, 2006) are among the most sensitive assays techniques (Queval et al., 2008). Despite the development of quantitative H_2O_2 assays, the results reported for H_2O_2 content in plant and fruit tissues vary significantly among species and relative cultivars, which is a result of the different sensitivity of the various methodologies applied and interference from other redox-active compounds (Queval et al., 2008). In fruit tissues, reported H_2O_2 content ranged from nmol/g FW to μmol/g FW. For example, Rao et al. (1998) measured 20–70 nmol/g FW of H_2O_2 in apples using the titanium H_2O_2 assay, while Vilaplana et al. (2006), using the Bioxytech H_2O_2-560 colorimetric assay, measured 5–25 nmol/g FW, which was similar to the 10–20 nmol/g FW reported by Torres et al. (2003). In pears, 0.5–0.8 μmol/g FW of H_2O_2 was reported by Brennan and Frenkel (1977) using the titanium H_2O_2 assay, while 6–11 nmol/g FW was reported using the Bioxytech H_2O_2-560 kit (Lentheric et al., 2003). In strawberry fruit, 20–90 μmol/g FW of H_2O_2 was reported (Woods et al., 2005). Although, colorimetric and spectrometry are the most popular used methods, they are prone to interferences of color and polyphenols which affect the accuracy of measurement. A simple, quantitative and reliable protocol suitable for fruit tissue is needed.

The luminol CL method developed by Warm and Laties (1982) based on the ferricyanide catalysed oxidation of luminol by H_2O_2 has been widely used (Queval et al., 2008). The replacement of ferricyanide, the traditional catalyst of luminal luminescence by Co (II), enhanced the sensitivity of the reaction with H_2O_2 by three orders of magnitude (Yuan and Shiller, 1999). However, due to the existence of high concentration of phenols and ascorbic acid, which quench luminescence in plant extracts, especially in fruit tissues, addition of PVP and ascorbate oxidase are required in the extraction procedures (Warm and Laties, 1982; Veljovic-Jovanoic et al., 2002). Recently, a modified protocol involving dilution of extracts to eliminate the quenching effects was reported. This protocol was reported to measure H_2O_2 at nanomole levels in grape leaves (Perez and Rubio, 2006). The reported procedure was simple, sensitive and suitable for a variety of plant tissues. However, this method was developed using leaf tissues and not tested on fruit (Cheeseman, 2006; Zhou et al., 2006), when this protocol was applied to 'Red Delicious' apple fruit tissues, it failed to detect any H_2O_2. Apple fruit peel contains interfering compounds such as anthocyanins, polyphenols and ascorbate, which may quench H_2O_2 in the measuring system, although 5% PVP was applied as described by Perez and Rubio (2006). Meanwhile, a simple colorimetric method involving activated charcoal to remove the interfering compounds was reported by Zhou et al. (2006). However, some H_2O_2 can also be removed by activated charcoal resulting in 32–40% loss.

The objectives of this study were to develop a modified chemiluminesence technique for apple and other fruit tissue by examining the efficiency of PVP, PVPP and charcoal to remove the interference compounds in the measuring system; establish optimal chemiluminesence measuring conditions for determining H_2O_2 in apple fruit tissues under various physiological conditions; and compare the developed chemiluminesence technique with the OXIS kit. This study provides optimized protocol for universal chemiluminescence (CL) method for H_2O_2 determination in various plant tissues which uses PVPP for removing contaminants.

2. Materials and methods

2.1. Plant materials

Apple fruit (*Malus x domestica* Borkh) 'Red Delicious' were harvested twice from two commercial orchards before the climacteric stage, with internal ethylene concentration 0.1–0.2 ppm (1 week before commercial harvest) and 1 week after commercial harvest. After harvest, fruits were divided into three groups, control, DPA treatment and 1-MCP treatment. DPA was applied by drenching the apple fruit in DPA solution (2000 μl/L) for 2 min and dried with paper towels. 1-MCP treatment was conducted in a sealed stainless steel chamber at concentration of 1.0 μl/L for 16 h. After treatments, fruit were stored in air at 0–1 °C for 5 months. 'Golden Delicious' and 'Cortland') were harvested in commercial orchards at commercial maturity and stored at 0 °C under CA conditions (2% O_2 + 1.0% CO_2) until they were used.

Strawberry fruit, spinach and corn leaves were grown in the growth chamber at the Atlantic Food and Horticulture Research Center, AAFC.

2.2. Reagents

The hydrogen peroxide solution, 30% (w/w), luminol (5-amino-2, 3-dihydro-1,4-phthalazinedione), PVP (polyvinylpyrrolidone), PVPP (polyvinylpolypyrrolidone), sodium carbonate, 3-amino-phthalhydrzide, catalase from bovine liver and cobalt (II) chloride hexahydrate were all purchased from Sigma–Aldrich Canada Ltd. (Oakville, Ontario). Trichloroacetic acid (TCA) was purchased from Fisher Scientific Canada (Ottawa, Ontario).

A 0.1 M sodium carbonate buffer was prepared and the pH was adjusted to 10.2. Stock luminol solution was prepared as described by Perez and Rubio (2006) by dissolving 11.5 mg luminol in 10 ml sodium carbonate buffer. Stock cobalt chloride was prepared by dissolving 7.14 mg in 10 ml sodium carbonate buffer. 5% TCA was prepared in water. A 50 U catalase solution was prepared in water. A stock mixed reagent was prepared by diluting 10 ml of the stock luminal and 2 ml of the cobalt chloride in 100 ml sodium carbonate buffer. This solution was stored at 4 °C in the dark for at least 1 h, diluted 10 times with buffer and stored at 4 °C in the dark for approximately 18 h before use. A stock solution of H_2O_2 standard was prepared by diluting 10 μl of the H_2O_2 (30%, w/w) in 1.0 ml 5% TCA. A series of standards were prepared from the stock ranging from 0.00677–0.10170 nmol H_2O_2 in 5% TCA. A full set of standards was run with each set of samples.

2.3. Measuring procedure

Plant tissue (peel, flesh, calyx or leaves) was frozen and ground to a powder in liquid nitrogen. Approximately 0.10 g of the frozen tissue was weighed in to a 2 ml centrifuge tube. 500 μl of 5% TCA was added and the homogenate was centrifuged at 13,000 × *g* for 12 min. Insoluble PVPP (5%), PVP (5%) or activated charcoal (AC) (12%) was added at this point and the sample was centrifuged at 13,000 × *g* for an additional 3 min. An aliquot of the samples was diluted 1000 times with 0.1 M carbonate buffer. 40 μl of the dilution was then incubated at 30 °C for 15 min with either 10 μl of 50 U catalase or distilled water. Following incubation, 10 μl of sample was transferred to the well of a 96 well plate (white microliter plate, Thermo Electron Corporation, cat # 9502887), 250 μl of the diluted reaction reagent was added to each well and the luminescence was measured over 8 s on a Fluoroskan Ascent FL (Thermo Electron Corporation, Mississauga, Ontario). The difference between the response for the catalase treated and non treated sample was considered specific for H_2O_2. The absolute specific CL of samples which has subtracted the background specific CL of blank was calculated from a standard curve equation obtained each time when the samples were analyzed and expressed as μmol/g FW. The freshly prepared mixed solution was stored in the dark for at least 1 h and then diluted 10 times, and stored in the dark for 12 h before used.