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Antioxidant activity and oxygen-scavenging system in orange pulp during fruit ripening and maturation

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Abstract

The antioxidant activity (ferric reducing/antioxidant power, FRAP) and oxygen-scavenging system of pulps during the fruit ripening and maturation were investigated in three cultivars of sweet orange (*Citrus sinensis* (L.) Osbeck). The highest FRAP values and activities of antioxidant enzymes and contents of nonenzymes were detected in 'Red Flesh' navel orange. The activities of oxygen-scavenging enzymes, superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (G-POD), decreased with ripening and maturation of fruit. Ascorbate peroxidase (AsA-POD) and dehydroascorbate reductase (DHAR) activities were also stably declined, whereas glutathione reductase (GR) activity and nonenzymes in the ascorbate–glutathione cycle displayed a single-peak pattern which paralleled the significant changes in FRAP values. In addition, the ratios of ascorbate/dehydroascorbate (AsA/DHAsA), reduced glutathione/oxidized glutathione (GSH/GSSG) were also decreased. Changes in the activities of antioxidant enzymes and the contents of nonenzymes during ripening indicated that the antioxidant system plays a fundamental role in the ripening of orange fruits.

Keywords: Antioxidant activity; Orange; Oxygen-scavenging enzymes; Ascorbate; Glutathione; Ripening and maturation

1. Introduction

Active oxygen species (AOS), mainly superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$) (Halliwell and Gutteridge, 1989), are constantly generated in vivo for physiological purposes, especially under pathological conditions, resulting in oxidative damage in plants. The AOS can rapidly attack all types of biomolecules to cause membrane deterioration, lipid peroxidation and DNA mutation, leading to metabolic and structural dysfunctions and cell death (Halliwell and Gutteridge, 1989). In order to decrease these biological damages for survival, all organisms have evolved a well-integrated antioxidant system, including enzymatic and non-enzymatic components. The antioxidative enzymes cover superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidases (G-POD) and the ascorbate–glutathione cycle enzymes: ascorbate peroxidase (AsA-POD), mono- and dehydroascorbate reductase (MDHAR, DHAR) and glutathione reductase (GR). The non-enzymatic antioxidants ascorbate (AsA) and glutathione (GSH), together with the enzymatic components, ultimately scavenge H_2O_2 at the expense of nicotinamide adenine dinucleotide phosphate (NADPH) or nicotinamide adenine dinucleotide (NADH) (Asada, 1999). It has been reported that formation of $O_2^{\bullet -}$ and accumulation of H_2O_2 were more obvious during ripening, as indicated by increase in lipid peroxidation and protein oxidation products (Jiménez et al., 2002). The AOS seems to be an intrinsic feature of senescence and fruit ripening since they could promote the process of oxidative deterioration that contributes to a general deterioration of cellular metabolism (Thompson et al., 1987; Del Río et al., 1998). It is likely, therefore, that the antioxidant systems, which are distributed in cell organelles (Castillo and Greppin, 1988; Foyer and Mullineaux, 1998; Jiménez et al., 1998a; Corpas et al., 2001), play an important role in both the senescence and the ripening process. For the foregoing reason, there must be a balance between biosynthesis of AOS and their removal by antioxidant systems. On the other hand, the rich sources of various antioxidant components were required by human body for optimal health. In the recent years, more attention has been paid to the antioxidants contained in fruits because epidemiological studies revealed that high fruit intake was associated

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with reduced mortality and morbidity of cardiovascular disease and some types of cancer and one of possible mechanisms was attributed to the antioxidant activity presented by the fruits.

Orange fruits attract great attention because of their nutritional and antioxidant properties, especially the component of ascorbate–glutathione cycle. Due to the importance of ascorbate–glutathione cycle, it is important to understand the evolution of its production and losses during fruit development, ripening and maturation. Changes in enzymatic and non-enzymatic components of the plant antioxidant defenses have been previously described during fruit ripening in several plants. For example, Jiménez et al. (2002) reported that the levels of GSH and AsA in tomato increased during the ripening process. However, different result has been provided by Wang and Jiao (2001), who found that GSH, AsA and related enzyme should decline with ripening of blackberry fruit. It is noted that information on antioxidant system is still lacking in orange. Such knowledge is needed as a basis to determine the ideal harvest dates in order to pick fruits with a maximum amount of ascorbate, and to determine ways to improve its production and to reduce their losses. To this aim, we carry out the present to determine the changes in antioxidant systems, enzymes in the ascorbate–glutathione cycle, and the role of these antioxidant systems in the degradation of membranes during maturation, ripening and senescence of oranges.

2. Materials and methods

2.1. Fruit sample preparation

Fruits of three sweet orange (*C. sinensis* (L.) Osbeck) varieties with the same phenology, 'Red Flesh' navel orange, 'Newhall' navel orange and 'Sanguine' orange, were sampled from Zigui county in Hubei, China at the middle of June (young fruit stage), July (green fruit stage), August (early stage of fruit expansion), September (later stage of fruit expansion), October (early stage of fruit color turning), November (later stage of fruit color turning), December (maturity stage). Thirty fruits similar in fruit quality, from 30 orange trees grown under identical condition, were randomly divided into three groups as three replications. These operations were completed in an hour. Pulps from equatorial part of the fruits were collected, frozen in liquid N_2 in half an hour and stored at −80 °C until use.

2.2. Measurement of total antioxidant activity by ferric reducing/antioxidant power (FRAP) assay

Five grams of fruit tissue (fresh weight, FW) was ground in a mortar with 5 ml distilled water. The homogenate was centrifuged at 6000 × *g* for 10 min, and the supernatant was immediately used for FRAP assay, following a procedure described by Benzie and Strain (1996). Briefly, FRAP reagent composed to 2.5 ml of a 10 mM TPTZ (2,4,6-tripyridy-*S*-triazine, Sigma) solution in 40 mM hydrochloric acid plus 2.5 ml of 20 mM ferric trichloride and 2.5 ml of 0.3 mM acetate buffer, pH 3.6 and was prepared freshly and warmed at 37 °C. Aliquots of 40 μl the supernatant were mixed with 0.2 ml distilled water and 1.8 ml FRAP reagent, followed by incubation at 37 °C for 10 min the absorbance at 593 nm via a spectrophotometer (SHIMADZU UV-2450). The 1.0 mM ferrous sulfate ($FeSO_4$) was used as the standard solution. The final result was expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1.0 mM $FeSO_4$. Adequate dilution was necessary if the FRAP value was beyond the linear range of standard curve.

2.3. Activity of SOD, CAT and G-POD assays

Five grams of fruit tissue (FW) was homogenized in 5.0 ml of 0.1 M phosphate buffer (pH 7.8). The homogenate was centrifuged for 10 min at 4200 × *g* (4 °C), and the resultant supernatant was used for SOD, G-POD and CAT assays.

SOD activity, expressed as Unit mg protein^{-1}, was measured using the methods of Monk et al. (1987). One SOD unit was defined as the amount of enzyme that inhibited 50% nitro blue tetrazolium (NBT) by light. G-POD activity, expressed as Unit mg protein^{-1}, was assayed according to a modified method based on Li (2000). A total volume of 3.0 ml reaction mixture containing 0.05 M potassium phosphate (pH 5.5), 2% H_2O_2, 0.05 M guaiacol and 100 μl of enzyme extract, was allowed to incubate for 3.0 min at 34 °C. The reaction was started by adding H_2O_2. G-POD activity was assayed by measuring the rate of guaiacol oxidation at 470 nm. One G-POD unit was defined as the amount of nanomoles of guaiacol oxidized per milligram of protein per minute. CAT activity was measured according to Kar and Mishra (1976). One unit of CAT was defined as the amount of enzyme, which decomposes 1 μmol H_2O_2 per minute at 25 °C.

2.4. Analysis to activity of AsA-POD, DHAR and GR

Five grams of fruit tissue (FW) was homogenized in 5.0 ml of 0.1 M Tris–HCl buffer (pH 7.8) containing 2.0 mM ethylenediaminetetracetic acid, disodium salt (EDTA-Na_2) and 2.0 mM dithiothreitol (DTT). The homogenate was centrifuged for 30 min at 20,000 × *g* (4 °C), and the supernatant was used for GR assay to Smith et al. (1988) with slight modification. One hundred μl of crude enzyme extract was added to a solution containing 0.05 M Tris–HCl buffer (pH 7.5), 3.0 mM magnesium chloride, 0.5 mM oxidized glutathione (GSSG), 2.0 mM ethylenediaminetetracetic acid (EDTA), 0.15 mM NADPH in a total volume of 1.0 ml. The activity of GR, expressed as nanomoles of NADPH oxidized per milligram of protein per minute, was determined by monitoring the glutathione-dependent oxidation of NADPH at 340 nm via a spectrophotometer (SHIMADZU UV-2450). The reaction was started by adding GSSG.

Five grams of fruit tissue (FW) was pulverized in a cold mortar and pestle with 5 ml of potassium phosphate buffer (0.1 M, pH 7.3) containing 1.0 mM EDTA and 2.0 mM DTT. The homogenate was centrifuged for 10 min at 12,000 × *g* (4 °C), and then used for AsA-POD and DHAR assays. AsA-POD activity, expressed as nanomoles of ascorbate oxidized per milligram of protein per minute, was determined based on a