Pak. J. Bot., 40(5): 2023-2029, 2008.

OXIDATION AND PEROXIDATION OF POSTHARVEST BANANA FRUIT DURING SOFTENING

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Abstract

Softening is a characteristic of fruit ripening caused by oxidative action. The oxidized degree of membrane lipids and proteins in relation to production of reactive oxygen species (ROS) of postharvest banana fruit during softening were investigated. Firmness as an indictor of softening of banana fruit was also measured. Banana fruit firmness decreased markedly after 4 days of storage, which indicated the occurrence of fruit softening. The contents of malondialdehyde (MDA) and protein carbonyl after 3 days and lipofuscin content after 4 days of storage increased markedly, which exhibited that the accumulation of lipofuscin appeared later than that of MDA and protein carbonyl which could be associated with the late softening stage. Hydroxyl radical level and hydrogen peroxide content decreased within 3 days and then increased significantly. However, production of superoxide anion radical decreased within 2 days, then maintained a low level, and finally increased significantly after 5 days of storage. It was apparent that the marked increases of hydroxyl radical and hydrogen peroxide contents of banana fruit appeared prior to the time of fruit softening and the accumulations of MDA, protein carbonyl and lipofuscin. This study suggested that enhanced production of hydroxyl radical and hydrogen peroxide could participate in the formation of oxidative products and then involve the initiation of banana fruit softening.

Introduction

The oxidative damage of plasma membranes and proteins are caused mainly by the reactive oxygen species (ROS) (Koc *et al*., 2004). ROS production occurs primarily as by-products of cellular metabolism in the mitochondria and normal mitochondrial respiration is associated with inevitable electron leak, resulting in a nonstop formation of ROS, such as superoxide anion radical, hydrogen peroxide and hydroxyl radical (Masaki *et al*., 1999; Terman *et al*., 2006). Excessive production of ROS related with respiratory climate can damage cellular composition such as proteins and lipids and leads to the loss of membrane integrity and functionality.

Enhanced peroxidation can damage membrane lipids and proteins. Protein-bound carbonyl has been considered a specific marker of protein oxidation (Abd *et al*., 2005; Grune *et al*., 2001). Lipofuscin-like pigments (LFP) as the final products of the autoxidation of molecular components of cells (Akeo *et al*., 1992; Gugiu *et al*., 2006), particularly, unsaturated lipids (d'Ischia *et al*., 1996), represents increased oxidative damage to cells (Terman & Brunk, 2002; Terman *et al*., 2007). Malondialdehyde (MDA) is also a product of lipid peroxidation (Hodges *et al*., 1999). Oxidative products of membrane lipids and proteins as indicated by the accumulation of lipofuscin and protein-bound carbonyl, may account for the damage of the membrane functions, which indicates that plant cells could lose the balance between production and scavenging of ROS.

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Fruit softening is a characteristic of fruit ripening and excessive production of ROS accelerates fruit ripening (Fry *et al*., 2001; Cheng *et al*., 2008). It is suggested that cellular oxidation and peroxidation was associated with the progress in fruit softening. To our best knowledge, the degree of the cellular oxidation and peroxidation of harvested fruits during softening was evaluated only by ROS production level and malondialdehyde content in the most study. Unfortunately, these studies did not involve the oxidative and peroxidative products of membrane lipids and proteins. In this study, ROS production levels in relation to the oxidative and peroxidative products of membrane lipids and proteins, lipofuscin and protein-bound carbonyl were measured. The relationship between level of cellular oxidation and peroxidation, and softening degree of harvested banana fruit was determined. This study can help understand and elucidate the mechanism of fruit softening based on the oxidation and peroxidation points.

Materials and Methods

Plant materials: Mature pre-climacteric fruit of banana (*Musa* sp. cv. Williams, Cavendish sub-group AAA) were obtained from a local commercial plantation near Guangzhou. Individual fingers were dipped for 2 min in 0.05% Sportak (a.i. prochloraz) fungicide solution to control disease and then air-dried at 25°C for 3 h. Fruits were selected for uniformity of weight and shape without visual defects, treated with 1000 mL/L propylene (a functional ethylene analog) for 16 h, placed at random into unsealed plastic bags (3 fruit fingers/bag) and then held for 7 days to ripen at 22°C.

Measurement of fruit firmness: Fruit firmness of pulp tissues from ten individual fruits was measured every day. According to the method of Duan (Duan *et al*., 2008), peel tissues from one side of banana fruit finger were removed and then measurements were conducted at three different points using a penetrometer (Model GY-1, Hangzhou Scientific Instruments, Hangzhou, China) fitted with a 4 mm diameter flat probe and recorded as Newton (N).

Analysis of H_2O_2 production: H_2O_2 was determined by the method of Schopfer (Schopfer *et al*., 2001). Pulp tissues (1 g) of banana fruit were homogenized with 3 mL of 0.02 M phosphate buffer (pH 6.0) for 30 min. The homogenized solution was centrifuged for 10 min at 1900 *g* and the supernatant was then collected. The supernatant (1 mL) was incubated for 5 min with 3 mL of 0.02 M phosphate buffer (pH 6.0) containing 5 μM scopoletin (Sigma Chemical Co.) and 3 mg/mL horseradish peroxidase (Boehringer Mannheim) in darkness at 25°C using a shaker. The decrease in fluorescence (the excitation wavelength of 346 nm and the emission wavelength of 455 nm) in the incubation medium was measured against the reagent blank solution as a reference. Fluorescent value was transformed into molar H_2O_2 concentration using a linear calibration curve.

Measurement of ·OH production: ·OH production was estimated as described by Halliwell (Halliwell *et al*., 1988). Pulp tissues (1 g) of banana fruit were homogenized for 30 min in 3 mL of 0.02 M phosphate buffer (pH 6.0) and then centrifuged with 1900 *g* for 10 min. The supernatant (1 mL) was incubated for 30 min in 1.5 mL of buffer (0.02 M pH 6.0 phosphate buffer) containing 20 mM 2-deoxy-D-Rib (Sigma Chemical Co.) at

25°C and then the formation of the breakdown product malondialdehyde was determined by mixing 0.5 mL of centrifuged incubation medium with 0.5 mL of 2-thiobarbituric acid (10 g/L in 0.05 M NaOH) and 0.5 mL of 2.8% (w/v) trichloroacetic acid. After the reaction solution was treated in boiling water for exactly 10 min, cooled in tap water, and clarified by centrifugation, the reaction product was measured fluorometrically (the excitation wavelength of 532 nm and the emission wavelength of 553 nm) against reagent blanks. ·OH production content was expressed as the relative fluorescent intensity.

Measurement of superoxide anion production: According to the method of Schopfer (Schopfer *et al*., 2001), pulp tissues (1 g) of banana fruit were homogenized for 30 min in 3 mL of 20 mM K-phosphate buffer (pH 6.0) and then centrifuged with 1900 g for 10 min. The supernatant (1 mL) was incubated for 1 h with 2 mL of 10 μM dihydroethidium (DHE) and 100 μM $CaCl_2$ at pH 4.75. To prevent light oxidation, samples were maintained in the dark prior to analysis. The reaction product was measured fluorometrically using the excitation wavelength of 488 nm and the emission wavelength of 520–615 nm and the peak value as the concentration of superoxide anion against the reagent solution and expressed as the fluorescent intensity.

Measurement of MDA content: MDA content was determined by the method of Hodges (Hodges *et al*., 1999). Pulp tissues (1 g) of banana fruit were extracted for 2 hour with 5 mL of 10% (w/v) 2,4,6-trichloroanisole. The crude extract was centrifuged at 10000 *g* for 10 min., and then the supernatant was collected. An aliquot (1 mL) of the supernatant was vortexed with 4 mL of 20% (w/v) 2,4,6-trichloroanisole containing 0.5% (w/v) thiobarbituric acid, treated for 30 min., in a boiling water, cooled for 5 min., in ice and then centrifuged for 20 min., at 1640 *g*. The supernatant was collected and then the absorbances at 600 and 532 nm were measured, respectively. The MDA equivalent was calculated by the method of Dhindsa (Dhindsa *et al*., 1981) using the extinction coefficient of 155 mM cm^{-1}.

Measurement of lipofuscin: Extraction and determination of lipofuscin **(**LFP) were conducted by the method of Schutt (Schutt *et al*., 2002). Pulp tissues (1 g) were extracted for 30 min., with 3 mL of 0.05 M phosphate-buffer (pH 8.0) containing 20 mg/mL 2,6-ditertiarybutyl-4-methyl phenol. The resulting homogenate was shaken for 10 min at 45°C, and then centrifuged at 5000 g for 10 min. The supernatant phase was collected and an aliquot of the supernatant was passed through a filter membrane (Ø=0.45 μm). Lipofuscin content of the filtered solution was assessed by the relative fluorescent intensity at the excitation wavelength of 390 nm and the emission wavelength of 530 nm.

Estimation of protein oxidation: Content of protein carbonyl as an index of protein oxidation was estimated by the method of Uchida & Stadtman (Uchida & Stadtman, 1993). In brief, pulp tissues (1 g) of banana fruit were homogenized for 30 min in 3 mL of 0.02 M phosphate buffer (pH 7.0) and then centrifuged for 10 minutes at 1900 *g*. The supernatant phase was collected and 0.8 ml of the supernatant was incubated with 0.8 mL of 0.1% (w/v) 2, 4-dinitrophenyl hydrazine (2,4-DNPH) in 2 M HCl. In this study, 2 M HCl was used as a control. After 60 min., of incubation at room temperature, 2 mL of 20% trichloroacetic acid was added, mixed and then centrifuged for 10 min., at 1900 *g*. After washing by ethanol/ethylacetate mixture (50:50, v/v), the residue was dissolved

into 3 mL of 8 M guanidine hydrochloride in 133 mM Tris solution (pH 7.2) containing 13 mM ethylenediaminetetraacetic acid and then centrifuged at 1900 *g* for 10 min. The supernatant phase was collected and then the absorbance of the supernatant was recorded at 365 nm using an UV/VIS spectrophotometer (ELICO, Model SL 159) against the control solution. Results were expressed as l mol of 2,4-DNPH-incorporated amount per microgram of protein based on a molar extinction coefficient of 2.1×10^4 M^{-1} cm^{-1} for aliphatic hydrazones.

Protein content determination: Protein content was determined by the method of Lowry (Lowry *et al*., 1951) with bull serum albumin as the standard.

Statistical analysis: Data were expressed as means ± standard deviations of three replications. The significant differences between means within the confidence interval of 95% were analyzed by *t*-test.

Results and Discussion

Decrease in fruit firmness is a characteristic of fruit softening ripening (Jiang *et al*., 2004). In this study, banana fruit firmness decreased gradually during storage and a significant reduction in the firmness was observed after 4 days of storage (Table 1), which indicated the occurrence of fruit softening. As firmness decreased markedly, banana fruit became soft and skin turned to be yellow.

MDA is a product of lipid peroxidation (Hodges *et al*., 1999) while protein-bound carbonyl has been considered a specific marker of protein oxidation (Abd *et al*., 2005; Grune *et al*., 2001) and lipofuscin-like pigments (LFP) as the final products of the autoxidation of molecular components of cells (Akeo *et al*., 1992; Gugiu *et al*., 2006) can account for the oxidative extent (d'Ischia *et al*., 1996;, Terman & Brunk, 2002). Siu *et al*., reported that MDA, lipofuscin, and protein oxidation provided an index of cell oxidized damage (Siu *et al*., 1999) and oxygen-derived free radicals could cause cellular oxidation and consequent membrane lipid peroxidation (Siu & To, 2002). Thus, membrane stability can be evaluated well by levels of MDA, lipofuscin and protein oxidation during fruit ripening / softening. In this study, contents of MDA, protein carbonyl, and lipofuscin of banana fruit tended to increase during storage. The contents of MDA and protein carbonyl of banana fruit after 3 days and lipofuscin content after 4 days of storage increased markedly, which indicated that the accumulation of lipofuscin appeared later that of MDA and protein carbonyl, and could be associated with the late softening stage. Furthermore, the increased level of the oxidation and peroxidation in harvested banana fruit may account for the senescence in the period of fruit softening. The study exhibited further that a relationship existed between the oxidation and peroxidation, and banana fruit softening.

The oxidation and peroxidation of membrane lipids and proteins could be caused by ROS (Koc *et al*., 2004). Production of ROS such as superoxide anion radical, hydrogen peroxide and hydroxyl radical production occurs primarily as by-products of cellular metabolism in the mitochondria and normal mitochondrial respiration (Masaki *et al*., 1999; Terman *et al*., 2006) and is considered as an important cause of cellular oxidation and consequent membrane lipid peroxidation, linking to fruit maturation and aging (Esterhazy *et al*., 2008). As shown in Table 2, various levels of hydroxyl radical, hydrogen peroxide and superoxide anion radical of banana fruit during storage were observed hydrogen peroxide and hydroxyl radical contents decreased within 3 days, then increased significantly, and finally decreased after 5 and 6 days of storage, respectively.

Table 1. The changes of firmness, MDA content, protein carbonyl content and lipofuscin content during the storage of banana fruits.

Days of storage	Firmness N	MDA content (nmol g^{-1} FW)	Protein carbonyl content (nmol mg^{-1} protein)	Relative fluorescent intensity of lipofuscin
1	11.228 ± 0.188a	4.645 ± 0.271c	4.751 ± 0.181d	7.160 ± 0.126d
2	11.071 ± 0.223a	4.025 ± 0.405c	5.441 ± 0.086c	6.561 ± 0.128f
3	11.071 ± 0.179a	8.743 ± 0.201c	5.830 ± 0.124b	6.531 ± 0.272f
4	10.028 ± 0.164b	24.278 ± 0.674b	7.370 ± 0.028b	6.814 ± 0.150e
5	7.257 ± 0.143c	26.477 ± 0.658b	7.477 ± 0.129b	8.004 ± 0.197c
6	5.414 ± 0.063d	54.503 ± 0.751a	7.750 ± 0.251b	9.146 ± 0.229b
7	3.342 ± 0.083e	51.778 ± 1.438a	8.452 ± 0.017a	11.110 ± 0.086a

Different letters within the same column indicate significant differences at 5% level.

Table 2. The changes of hydroxyl radical, hydrogen peroxide and superoxide anion radical content during the storage of banana fruits.

Days of storage	Relative fluorescent intensity of hydroxyl radical	Hydrogen peroxide content (mmol H_2O_2 g^{-1} FW)	Relative fluorescent intensity of superoxide anion radical
1	11.711 ± 0.144a	4.256 ± 0.207b	1.457 ± 0.049b
2	9.303 ± 0.177d	3.066 ± 0.188e	1.073 ± 0.058d
3	8.397 ± 0.117e	2.341 ± 0.068f	1.060 ± 0.058d
4	9.390 ± 0.140c	3.908 ± 0.080c	1.056 ± 0.034d
5	10.550 ± 0.198b	4.529 ± 0.077a	1.052 ± 0.072d
6	14.152 ± 0.288a	3.419 ± 0.150d	1.263 ± 0.074c
7	13.640 ± 0.183a	3.122 ± 0.087e	1.836 ± 0.032a

Different letters within the same column indicate significant differences at 5% level.

However, production of superoxide anion radical decreased within 2 days, then maintained a low level, and finally increased significantly after 5 days of storage. It was apparent that the significant increase in hydroxyl radical and hydrogen peroxide levels of banana fruit during storage appeared prior to the time of fruit softening and the accumulations of MDA, protein carbonyl and lipofuscin (Tables 1 and 2), which indicated that enhanced production of hydroxyl radical and hydrogen peroxide participated in the formation of oxidative products (Pennathur *et al*., 2005) and could involve in the initiation of fruit softening. At the later stage of fruit softening, increased production of superoxide anion radical might be related to decreased superoxide dismutase activity while reduced levels of hydroxyl radical and hydrogen peroxide could be due to loss of mitochondrial membrane potential and activity (Wiseman *et al*., 2007) responsible for cellular membrane damage through the formation of hydroxyl radical reaction with reducing iron in cytoplasm (Geracitano *et al*., 2005). Thus, this study help understand fruit softening involving ROS production, and the oxidation and peroxidation indicated by MDA, protein-bound carbonyl and lipofuscin-like pigments.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (Grant Nos. 30425040, 30500353, 30430490, 30371007 and U0631004) and Guangdong Provincial Natural Science Foundation (No. 06200670).

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(Received for publication 11 March 2008)