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Changes in ethylene production, carbohydrase activity and antioxidant status in pepper fruits during ripening

Chung Keat Tan [a], Zainon Mohd Ali [a], Zamri Zainal [a,b,*]

[a] School of Bioscience and Biotechnology, National University of Malaysia, 43600 Bangi, Selangor, Malaysia
[b] Institute of System Biology (INBIOSIS), National University of Malaysia, 43600 Bangi, Selangor, Malaysia

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ABSTRACT

The aim of the present study was to evaluate the changes in ethylene production, carbohydrase activities and antioxidant status in pepper Kulai at different ripening stages. This cultivar of pepper fruits exhibits a peak in ethylene production during stage 3 of ripening. The production of 1-aminocyclopropane-1-carboxylic acid (ACC) and ACC oxidase activity also peaked during stage 3, whereas ACC synthase activity increased gradually during ripening. In the carbohydrase activity study, the β-galactosidase activity increased significantly, whereas the α-mannosidase and α-galactosidase activities remained low and fairly constant throughout the ripening process. The lipoxygenase (LOX) activity, lipid peroxidation level and hydrogen peroxide content were also examined to evaluate changes in oxidation status. The LOX activity and hydrogen peroxide concentration decreased during ripening, whereas lipid peroxidation increased. However, the total antioxidant potential also increased, most likely as a defensive response towards oxidative stress. The activities of ascorbate peroxidase (APX) and glutathione reductase (GR) and the total phenolic content significantly increased during ripening. Furthermore, ascorbic acid levels greatly increased, and the ratio of reduced glutathione (GSH) to oxidised glutathione (GSSG) remained constant. However, the activities of superoxide dismutase (SOD) and catalase (CAT) declined. The overall results indicated that pepper Kulai is a climacteric fruit and that β-galactosidase activity increases as the fruit ripens and softens, suggesting a role for this enzyme in cell wall modification. In addition, the results presented here also revealed that the antioxidant capacity is enhanced during the ripening process and is accompanied by an increase in oxidative stress.

1. Introduction

Peppers have been a part of the human diet in the Americas since at least 7500 BC, and now they are widely used as a spice, food and medicine throughout the world. The widespread use of pepper is benefited by its natural colours and antioxidant compounds (Howard et al., 2000; Antonious et al., 2006). Epidemiological studies consistently indicate that there is an inverse correlation between the consumption of fruits containing antioxidants and the risk of human cancers, cardiovascular disease, diabetes and age-related declines in cognition (Knekt et al., 2002).

Pepper fruits are harvested and consumed at different stages of maturity, mainly the red and green stages, and they are sometimes consumed before they are completely ripe. Previously, peppers were harvested at the mature green stage when the pericarp becomes thick and the fruits reach their maximum sizes. However, in recent years, the mature red stage has become more favourable due to its improved flavour and nutritional aspects (Frank et al., 2005). During the ripening process, carbohydrates and vitamin C accumulate, and changes in the levels of total phenolic compounds and carotenoids have also been reported in some papers (Zhang and Hamauzu, 2003). Phenolic compounds are well known for their antioxidant qualities and their contributions to the sensory and nutritive qualities of the fruit, particularly the colour, taste, aroma and flavour (Tomás-Barberán and Espín, 2001).

The ripening of fruits is always accompanied by endogenous ethylene production and by the ability to respond to exogenous ethylene. In general, peppers are classified as a non-climacteric fruit based on the patterns of carbon dioxide and ethylene production, in addition to a transcriptome analysis (Lee et al., 2010). However, this finding is not conclusive and is limited to certain cultivars of *Capsicum*. Biles et al. (1993) have reported that the concentration of internal ethylene in New Mexican peppers is high enough to

* Corresponding author at: School of Bioscience and Biotechnology, National University of Malaysia, 43600 Bangi, Selangor, Malaysia. Tel.: +60 3 8925 5951; fax: +60 3 8921 2698.
E-mail addresses: jacktan0808_j2@hotmail.com (C.K. Tan), zainon@pkrisc.cc.ukm.my (Z.M. Ali), zz@ukm.my (Z. Zainal).

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initiate ripening. In addition, some cultivars of hot pepper have also demonstrated a peak in ethylene production during the ripening process (Gross et al., 1986).

Apart from ethylene production, fruit softening is also another common physiological change occur during ripening process. The fruit softening and textural changes are mainly due to differences in the cell wall thickness and composition (Harker et al., 1997). Previous studies have shown that pectin depolymerisation mediated by endo-polygalacturonase activity alone is not sufficient to significantly impact texture (Giovannoni et al., 1989). Other enzymes that are involved in cell wall metabolism have been identified in ripening fruit and, in some cases, have been tested for function. β-galactosidase (EC 3.2.1.23) may be one of the key enzymes involved. Gene expression study has indicated that there is a remarkable increase in β-galactosidase expression during the ripening process of tomato fruit (Smith and Gross, 2000). Down-regulation of tomato β-galactosidase results in decreased fruit softening (Smith et al., 2002). In addition to these and other enzymes, non-enzymatic factors such as reactive oxygen species (ROS) have also been identified that may play a role in cell wall degradation (Miller, 1986; Fry et al., 2001).

The pepper fruit has been identified as a potential solanaceous crop with extremely high antioxidant activity (Ou et al., 2002). A wide spectrum of antioxidant compounds, namely phenolic compounds, ascorbic acid, reduced glutathione (GSH)/oxidised glutathione (GSSG) and antioxidant enzymes, such as ascorbate peroxidase (APX; EC 1.11.1.11), superoxide dismutase (SOD; EC 1.15.1.1), glutathione reductase (GR; EC 1.8.1.7) and catalase (CAT; 1.11.1.6), are present in pepper fruits (Namiki, 1990). Recently, more attention has been paid to the role of natural antioxidants, particularly phenolic compounds. These compounds have shown positive but non-significant correlations ($R = 0.41–0.55$) with antioxidant activities (Al-Mamary, 2002) and may have more antioxidant activity than vitamins C and E and β-carotene (Vinson et al., 1995). Phenolic compounds reduce lipid autoxidation by inhibiting the initiation or propagation of oxidising chain reactions (Namiki, 1990).

The glutathione and ascorbate regulation cycle, along with SOD and CAT, constitute the major non-enzymatic and enzymatic systems that scavenge free radicals and H_2O_2 (Sala, 1998). Similarly to senescence, fruit ripening is accompanied by the deterioration of cell membranes (Ferrie et al., 1994). Lipoxygenase (LOX; EC 1.13.11.12) has been shown to have a central role in senescence-induced membrane deterioration by peroxidising free polyunsaturated fatty acids (Paliyath and Droillard, 1992). H_2O_2 may also be involved in the oxidative processes required in the initiation and promotion of fruit ripening (Brennan and Frenkel, 1977).

Formal studies involving pepper fruit have not been fully comprehensive and mostly concentrated on biophysical changes that occur during ripening. In this study, we examined the main physiological changes, which are ethylene production and carbohydrase activity involved in cell wall modifications during the ripening process. In addition, the evolution of the antioxidant status and the oxidative stress of peppers at different stages of maturity were also studied to improve the management and harvesting of this crop and to obtain fruit with higher nutritional values.

2. Materials and methods

2.1. Materials

Hot pepper fruits (*Capsicum annuum* cv. Kulai) were harvested at the Bukit Lancong Farm (Selangor, Malaysia). The selection was based on 5 different maturity stages: considerably mature green (stage 1; fully developed fruit with thick pericarp and dark green skin), 25% red (stage 2; skin of the fruit starting to turn red), 50% red (stage 3; approximately one-half green skin and the other half red), 75% red (stage 4; approximately 75% of the skin had turned red) and red (stage 5; fully ripe fruit). Overripe and damaged fruits were discarded. Fifty uniform fruits were selected for each stage of maturity and cut into small pieces, and the seeds were discarded. The samples were immediately frozen in liquid nitrogen and stored at −80 °C until use.

Unless otherwise stated, all solvents, salts and acids were purchased from Sigma Chemical Co. (St. Louis, USA). All of the reagents were of HPLC grade and were of the highest purity available. All aqueous solutions were prepared with distilled water.

2.2. Ethylene production

2.2.1. Ethylene measurement

Fresh fruits were used in this study. The ethylene production was measured by enclosing fresh fruits in an airtight container for 2 h at room temperature, withdrawing 1 mL of the headspace gas, and injecting this gas into an AutoSystem gas chromatograph (GC, PerkinElmer) fitted with a flame ionisation detector (FID).

2.2.2. ACC, ACC synthase and ACC oxidase assays

1-Aminocyclopropane-1-carboxylic acid (ACC) was extracted using the method of Sitrit et al. (1986) with slight modifications. The frozen tissues were homogenised with distilled water and centrifuged at 7000 rpm for 10 min. The supernatant was then used for determination of the ACC content based on the method described by Lizada and Yang (1979). A 100 μL aliquot of the aqueous pepper extract was added to a test tube with 100 μL of 5 μM $HgCl_2$ and 500 μL of distilled water. The test tubes were then sealed with serum caps, and 0.2 mL of an ice-cold mixture (2:1, v/v) of commercial bleach (5% NaOCl) and saturated NaOH was injected. The assay tubes were then vortexed and incubated in an ice bath for 15 min. After incubation, 1 mL of gas was withdrawn for the measurement of ACC content using gas chromatography. The efficiency of the conversion of ACC to ethylene in each sample was determined by adding a known amount of ACC as an internal standard to a replicate assay tube. The ACC content was defined as nmol per gram of fresh weight.

The ACC synthase (ACS; EC 4.4.1.14) activity was determined using the method of Nakatsuka et al. (1997) with some modifications. Five grams of frozen tissue was homogenised with 0.1 M phosphate buffer (pH 8.5) that contained 30 mM ascorbic acid, 4 mM dithiothreitol (DTT), 30 μM pyridoxal phosphate (PLP), 2% polyvinylpyrrolidone (PVP) and 10% glycerol. The homogenate was then filtered using cheesecloth and centrifuged at 11,000 rpm for 20 min. A 2-mL aliquot of the aqueous extract was desalted and subjected to gel filtration using Sephadex G-25 (1.5 cm × 6 cm). One millilitre of the desalted eluent was added to 0.2 mL of 0.5 mM *S*-adenosylmethionine (*S*-AdoMet or SAM). The reaction mixture was incubated at 30 °C for 30 min. The ACC production was then assayed according to the method of Lizada and Yang (1979), as described above. The enzyme activity was defined as nmol of ACC produced in 1 h/g of fresh weight.

The ACC oxidase (ACO, EC 1.14.17.4) activity was also assayed according to the method of Nakatsuka et al. (1997) with slight changes. The eluent collected from the gel filtration was mixed with 50 μL of 20 mM ACC, 5 μL of 2 mM $FeSO_4$ and 100 μL of 30 mM $NaHCO_3$ in a test tube. The test tube was then sealed with a serum cap. The test tube was incubated at 30 °C with shaking for 30 min, and the amount of ethylene formed was determined as described above. The ACO activity was defined as nmol of ethylene produced per hour per gram of fresh weight.