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Adenylate quantitative method analyzing energy change in postharvest banana (*Musa acuminate* L.) fruits stored at different temperatures

Xuemei He[a,b], Li Li[a,b], Jian Sun[a,b,*], Changbao Li[a,b], Jinfeng Sheng[a,b], Fengjin Zheng[a], Jiemin Li[a,b], Guoming Liu[a], Dongning Ling[a], Yayuan Tang[a]

[a] Agro-Food Science and Technology Research Institute, Guangxi Academy of Agricultural Sciences, 174 East Daxue Road, 530007 Nanning, China
[b] Guangxi Crop Genetic Improvement Laboratory, 530007 Nanning, China

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ABSTRACT

Energy level is closely related to postharvest banana fruit senescence. In this study, high performance liquid chromatography (HPLC) method was developed to determine concentrations of adenylate, i.e. adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP), in postharvest banana fruits. Boiling water extracting adenylate from banana fruits was suitable because of its high extraction rate and stability. HPLC method exhibited good repeatability (variation coefficients of 1.51%–3.42%) and recovery rate (93.9%–97.8%). The correlation coefficients of ATP, ADP and AMP with peak areas in a range of 1–120 mg/L were 0.999965, 0.999995 and 0.999996, respectively. Through analyzing adenylate concentrations and membrane hydrolysis-related enzyme activities (phospholipase D and lipoxygenase, ab. PLD and LOX, two key enzymes catalyzing membrane lipid degradation) in banana fruits stored at different temperatures (7 °C, 14 °C and 25 °C), it could be found that appropriate low temperature (14 °C) delayed fruit senescence by maintaining high energy level. Chilly stress (7 °C) accelerated fruit senescence by declining energy supply level, accompanying by activation of membrane hydrolysis-related enzymes such as PLD and LOX. These results were helpful for elucidating relationship between energy metabolism and senescence regulation during chilling injury in postharvest banana fruits.

1. Introduction

Banana (*Musa acuminate* L.) is one of the major commercial fruit crops grown in tropics and subtropics, which plays a key role in the economy of developing countries. Banana is a typical respiration climacteric fruit with short storage time. Low temperature storage is the most common method for preserving fruits and vegetables, but can not be used in banana preservation because of its sensitivity to low temperature (chilling injury and quality reduction occur below 12 °C) (Jiang et al., 2004; Yang et al., 2009). The sensitivity to low temperature becomes the biggest obstacle for cold chain logistics of banana fruits. Energy metabolism is an important metabolic pathway in physiology and biochemical reaction, which can be used to indicate the energy state of tissues and cells (Liu et al., 2011; Wang et al., 2014). Adenosine triphosphate (ATP) is the energy source of biological organism and plays a key role in cell metabolism, because energy metabolism, storage and utilization take ATP as the center (Manganaris et al., 2008). Adenylate is the general term for ATP, adenosine diphosphates (ADP) and adenosine monophosphate (AMP). Adenylate energy charge (EC) regulates the metabolic activities of organization, calculated as $EC = ([ATP] + 0.5 \times [ADP]) \times 100/([ATP] + [ADP] + [AMP])$. Cell energy is a vital factor to control ripening and senescence of postharvest fruits. Considerable evidences have suggested that ATP content and EC significantly increase in developing preharvest fruits and then decline during postharvest senescence. Preharvest application of boron (B) and calcium (Ca) (Xuan et al., 2005), and postharvest treatments such as high oxygen (Duan et al., 2004), ethylene inhibitor (Qu et al., 2006), controlled atmosphere storage (Harb et al., 2006), exogenous carbon source and ATP (Song

* Corresponding author at: Agro-Food Science and Technology Research Institute, Guangxi Academy of Agricultural Sciences, 174 East Daxue Road, 530007 Nanning, China.
E-mail address: jiansun@gxaas.net (J. Sun).

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et al., 2006), can maintain tissue levels of ATP and EC, thereby delaying pericarp browning of litchi and longan as well as internal flesh browning of "Conference" pear. Some studies have shown that senescence and chilling injury occurrence relates to the lack of energy supply in fruits and vegetables, and high ATP and EC levels effectively alleviate chilling injury and senescence symptom (Jian et al., 1999; Sondergaard et al., 2004; Ghasemnezhad et al., 2008; Acevedo et al., 2013; Vanlerberghe, 2013; Jin et al., 2014).

Chilling injury is a manifestation of senescence. Some studies have shown that chilling injury occurrence relates to the lack of energy supply in fruits, and high ATP and EC levels effectively alleviate chilling injury and senescence symptom (Jian et al., 1999; Sondergaard et al., 2004; Ghasemnezhad et al., 2008; Acevedo et al., 2013; Vanlerberghe, 2013; Jin et al., 2014). Cell membrane gets the first damage in fruits under chilling injury. Energy level plays an important role in maintaining cell membrane integrity. The lack of energy destroys membrane integrity by affecting the metabolism of membrane components. Phospholipase D (PLD) is a key enzyme in lipid degradation which plays an important role in cell membrane degradation process under aging and cold stress. Lipoxygenase (LOX) also includes the oxidation of polyunsaturated fatty acids and their corresponding lipids, resulting in membrane damage (Wang, 2001; Mao et al., 2007). More and more studies have suggested that a sufficient available energy status and a stable membrane lipid degradation enzymatic system in fruits collectively contribute to inhibit senescence in long-term cold storage. Treatment with exogenous ATP supply can maintain the higher energy levels and inhibit the activities of membrane hydrolysis-related enzymes (PLD and LOX), so as to reduce membrane lipid peroxidation and preserve membrane integrity of harvested litchi fruit and cut carnation flowers (Yi et al., 2008; Song et al., 2006; Song et al., 2008). Cell energy directly affects membrane lipid formation and cell membrane repair so as to regulate the cold tolerance of fruits under low temperature stress, which has been confirmed in cucumber, mango and peach (Mao et al., 2007; Jin et al., 2013; Jin et al., 2014). However, the reports about the effects of different storage temperatures on energy metabolism and membrane hydrolysis-related enzymes are absent for postharvest banana fruits.

The structures and biochemical properties of ATP, ADP and AMP are very similar, so it is hard to separate and quantify them. In recent decades, a small amount of literatures have reported that high performance liquid chromatography (HPLC) can be chosen to determine adenylate contents in fish and litchi tissues (Ryder, 1985; Veciana-Nogues et al., 1997; Yi et al., 2010). This method increases detection sensitivity and accuracy, while it exhibits a lot of disadvantages. The combination of organic phase (e.g. methanol and acetonitrile) and phosphate inorganic phase significantly shortens determination time but easily causes overlapping peaks (Lee et al., 2005; Li et al., 2014). Meanwhile, the use of high concentration phosphate is prone to clogging chromatographic column, and needs long time to clean column after mensuration. Therefore, a suitable method for analyzing adenosine phosphate in banana tissues is essential and necessary.

The present study is to develop a simple and suitable extraction and quantization method for ATP, ADP and AMP in postharvest banana fruits. By this method, energy change levels in banana fruits stored at different temperatures were determined, and the effects of energy metabolism on fruit senescence response to cold stress were further revealed. In addition, the activities of membrane hydrolysis-related enzymes which coordinated with energy metabolism to regulate senescence were also analyzed when banana fruits were stored at different temperatures. These results are important to research on postharvest banana preservation by regulating energy metabolism.

2. Materials and methods

2.1. Materials and reagents

Banana (cv. Guijiao No. 6, a mostly planting cultivar in China) fruits were picked at commercial maturity in an orchard of Nanning, Guangxi Province, China. After harvest, these fruits were immediately transported into a laboratory at Guangxi Academy of Agricultural Sciences. Banana fruit samples without mechanical damage were screened for uniform maturity and size. ATP, ADP and AMP standards, perchloric acid, potassium hydrogen phosphate, potassium dihydrogen phosphate, tetrabutylammonium hydrogen sulfate and acetonitrile (HPLC grade) were purchased from Sigma Chemical Co. (Saint Louis, MO, USA). All reagents were dissolved in deionized water, and then filtered with the Sybron/Barnstead Millipore system and a 0.45 μm filter (Merck MILI Bo, Billerica, MA, USA).

2.2. Extraction of ATP, ADP and AMP from postharvest banana fruits

Adenylate was extracted from postharvest banana fruits using two methods, i.e. perchloric acid extraction and boiling water extraction. Banana fruits were cut into small pieces, rapidly frozen in liquid nitrogen, and homogenized into powder.

Perchloric acid extraction: ATP, ADP and AMP were extracted by perchloric acid according to the modified method of Liu et al. (2006). Two grams of banana powder were weighed and extracted for 1 min with 10 mL of 0.6 M perchloric acid in an ice bath. The extraction mixture was centrifuged at 12,000 × *g* for 15 min at 4 °C. Six milliliters of supernatant were quickly neutralized (pH 6.5–7) with 1 M KOH, then diluted to 10 mL, passed through a 0.45-μm filter, and used for ATP, ADP and AMP measurements.

Boiling water extraction: Banana powder (2 g, prepared above) was weighed and extracted for 5 min with 10 mL of boiling water. The extraction mixture was centrifuged at 12,000 × *g* for 15 min the supernatant was diluted to 10 mL, passed through a 0.45-μm filter, and used for ATP, ADP and AMP measurements.

2.3. Preparation of standard stock solutions and HPLC analysis

ATP, ADP and AMP standards were dissolved in deionized water to obtain 100 mg/mL ATP, ADP and AMP standard stock solutions, respectively. Aliquots of the stock standard solutions were diluted to concentrations of 0, 0.2, 0.5, 1, 2 and 4 mg/mL in deionized water. A volume of 20 μL of each sample was taken for HPLC analysis using a Waters 2695 HPLC system (Waters Corporation, Milford, MA, USA) equipped with a Hubble C18 column (5 μm × 250 mm × 4.6 mm, OMNI, NC, USA) and a PDA detector. Column temperature was maintained at 40 °C. Standards and samples were separated using a gradient mobile phase. Mobile phase A consisted of 20 mM K_2HPO_4 and 5 mM tetrabutylammonium hydrogen sulfate dissolved in deionized water and adjusted to pH 7 with 0.1 M KOH. Mobile phase B was acetonitrile. The linear gradient conditions were as follows: 0 min, 95% A and 5% B; 5.5 min, 80% A and 20% B; 8 min, 70% A and 30% B; 14 min, 95% A and 5% B. Flow rate was set at 1 mL/min, and injection volume was 20 μL. Detection wavelength was set at 258 nm. ATP, ADP and AMP in samples were identified by comparison with retention time of standards, while ATP, ADP and AMP concentrations were determined using the external standard method. Data were expressed as means of six replicate determinations.