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Analysis of resistant starch degradation in postharvest ripening of two banana cultivars: Focus on starch structure and amylases

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ABSTRACT

Bananas are well known as good sources of dietary energy, with high levels of sugar and starch. To further analyze the starch-degradation mechanism in bananas, two species, Cavendish and Plantain, were used to investigate the influences of hydrolases and granule structure on starch degradation. Determination of the levels of resistant starch (RS), non-resistant starch (non-RS), total starch, and amylose content showed that each starch component content decreased gradually during the fruit-ripening process in both Cavendish and Plantain. Compared to Cavendish, Plantain had a higher content of total starch and RS, a faster starch-degradation rate, and a lower decrease in the ratio of RS/total starch. Scanning electron microscopy (SEM) images revealed that the starch granules of Cavendish were larger and more rounded than the smaller and ellipsoidal starch granules found in Plantain. Also, the analysis of gene expression suggested that β-amylases make a central contribution to starch degradation in both species and highly up-regulated β-amylases were correlated with the faster starch-degradation rate in Plantain. Two α-amylases, one starch phosphorylase, and one starch debranching enzyme were specifically up-regulated in Plantain, which might hydrolyse more non-RS compared with Cavendish.

1. Introduction

Starch is a product of photosynthesis and present semi-crystalline in plant cell plastids. In animals, starch is digested at different rates according to the structure of the starch in the starch granules. There are three categories: 1. rapidly digestible starch, which can be digested and absorbed orally and in the small intestine rapidly (digestion time < 20 min); 2. slowly digestible starch, which can be digested in the small intestine (digestion time between 20 and 120 min); 3. resistant starch (RS), which refers to the starch that cannot be digested or absorbed in the small intestine (digestion time > 120 min). The existance of resistant starch was first proposed by Englyst et al. (1982). The physiological function of RS is similar to dietary fiber, in that it can delay blood glucose and insulin response, and reduce the concentration of cholesterol and triglycerides. Resistant starch is fermented and degraded to short-chain fatty acids that acidify the intestine contents and may play a role in the prevention of colon cancer (Fuentes-Zaragoza et al., 2010). As more attention is being paid to healthy diets, RS has become a focus for research activity.

Bananas (*Musa* spp.) are well known as good sources of dietary energy, with high levels of sugar and starch (Zhang et al., 2005). It has been reported that green bananas are rich in RS (>40% dry weight [DW]), containing even more RS than traditional food crops such as rice and wheat (Goñi et al., 1996). In green bananas, the ratio of RS/total starch is more than 60% (Agama-Acevedo et al., 2014). However, the RS content dramatically decreases during the banana fruit-ripening process (Soares et al., 2011). Therefore, further exploration of the regulatory mechanisms of RS metabolism in bananas during fruit ripening is not only important for quality improvement, but also provides valuable guidance for banana fruit processing in banana ripening factories. However, little information is currently available about the biochemical mechanisms and genetic control of RS degradation in bananas.

"Banana" is also used as the common name for the plants which produce the fruit. The two major subgroups of banana are Cavendish and Plantain, which are both are widely cultivated, and are distinguished according to genotype. The Cavendish subgroup is the most widely grown group of bananas, since it includes the cultivars that dominate the international trade in bananas and is mainly consumed as a dessert. The Plantain subgroup refers to a set of more than 100 cultivars of cooking bananas that display a wide range of morphological variability. The amounts of RS are influenced by different banana cultivars (Eggleston et al., 1992) and the degree of ripeness (Cordenunsi

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and Lajolo, 1995). Furthermore, significant differences in the microstructure and physicochemical characteristics of starch granules were revealed among the banana RS samples during different ripening stages (Wang et al., 2014). Biochemical analyses revealed that a series of enzymes participated in starch degradation (Smith et al., 2005). Those enzymes can be classified into five categories, mainly including β-amylases, α-amylases, phosphorylases, debranching enzymes, and transporters. However, little information is available on the relationship between the different starches, enzymes and genes involved in the starch metabolism processes occurring during ripening.

In this work, two banana species (Cavendish and Plantain) were chosen to analyze the differences in their starch-degradation profiles during the ripening process. The contents of RS, non-RS, total starch, and amylose were determined. Scanning electron microscopy (SEM) was used to investigate the shapes and sizes of the granules during ripening. The expression levels of amylases, phosphorylases and starch debranching enzymes were estimated by real-time quantitative reverse transcription PCR (qRT–PCR).

2. Materials and methods

2.1. Fruit materials preparation

Cavendish (*Musa acuminata* L. AAA group, cv. Brazilian) and Plantain (*Musa acuminata* L. AAB group, cv. Obino l'Ewai) banana fruit were obtained from the Institute of Fruit Tree Research, Guangdong Academy of Agricultural Sciences, at the mature green stage (100–110 d after flowering). Fruit fingers were selected for uniformity of shape, size, and color. After ethylene treatment (500 $\mu L\,L^{-1}$) for 16 h, fruit was stored at 24 °C and 90% moisture. The samples were collected at 0, 2, 3, 4, and 5 d in storage time, and subsequently frozen in liquid nitrogen and stored at −80 °C prior to further analysis. Five banana fingers were selected for each replicate. All samples were prepared with at least three biological replicates.

2.2. Resistant starch (RS) and non-resistant starch (non-RS) content determination

Fruit flesh (0.5 g fresh weight) was ground into powder with liquid nitrogen using mortar and pestle, then treated with successive washes of 80% alcohol, 50% alcohol, and water to remove soluble sugar and other soluble substrates. Resistant starch and non-RS contents were analyzed using a Resistant Starch Assay Kit (Megazyme, Bray Business Park, Bray, Wicklow, Ireland) according to the manufacturer's instructions. Total starch content is the sum of RS and non-RS.

2.3. Isolation of starch granules

Starch granules were isolated from the fruit pulp at five ripening stages using a reported protocol (Soares et al., 2011) with the following modifications. Flesh pulp was ground into powder with liquid nitrogen then a 5 g sample was directly suspended in 0.005 L of pectinase solution (15 $g\,L^{-1}$, pH 4.0, Sigma Chemical Co.) for enzymic removal of cell walls. The homogenate was laid in a shaking water bath at 45 °C for 2 h, then filtered using Miracloth membrane (Calbiochem). After being centrifuged at 3000*g* for 10 min, the pellet was washed with distilled water three times. The pellet was dried in a drying oven, and stored at room temperature for SEM and amylose content analysis.

2.4. Content of amylose analysis

Amylose content was determined using a two-wavelength method with potassium iodide (Zhu et al., 2008). Dry starch granules (0.1 g) were completely dissolved in 0.01 L potassium hydroxide solution (0.5 $mol\,L^{-1}$), then diluted to 0.6 L with distilled water; 0.03 L were titrated to pH 3.5 with hydrochloric acid (0.1 $mol\,L^{-1}$), then 0.002 L iodine solution was added. After color development, samples were scanned at 624 and 472 nm using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Results were evaluated using the method described by Zeng et al. (2012).

2.5. Scanning electron microscopy (SEM) analysis

The dried starch granules were fixed onto stubs using double-sided tape and coated with a 10 nm thick platinum layer using the JEOL JFC-1600 (JEOL, Tokyo, Japan) coating system. The samples were examined on a JEOL JSM-6360LV (JEOL, Tokyo, Japan) scanning electron microscope. Scanning electron microscopy was performed in secondary electron mode at 15 kV. After SEM analysis, the images were loaded in SmileView (JEOL Ltd, Tokyo, Japan) software. The length of starch granules was measured using "length measurement" base on scaleplate value. Fifty particles were measured for each sample and the average particle size of the starch nanoparticles was determined.

2.6. RNA isolation

Total RNA was extracted from fruit flesh according to Asif et al. (2000) with the following modifications. A 6 g sample was added to preheated (65 °C) extraction buffer (containing 2.5% CTAB, 0.1 L Tris-HCl pH 8.0, 1.4 $mol\,L^{-1}$ NaCl, 0.02 $mol\,L^{-1}$ EDTA, pH 8.0), then incubated in a water bath at 65 °C for 30 min, an equal volume of chloroform added, then mixed and centrifuged at 12,000 x *g* for

Table 1
Resistant starch (RS) and non-resistant starch (non-RS) contents in pulp of Cavendish and Plantain at five ripening stages.

Starch	Species	0 d	2 d	3 d	4 d	5 d
RS	Cavendish	0.181 ± 0.007 b	0.114 ± 0.008 c	0.075 ± 0.004 d	0.057 ± 0.003 e	0.027 ± 0.002 f
	Plantain	0.253 ± 0.011 a	0.201 ± 0.026 b	0.140 ± 0.015 c	0.080 ± 0.008 d	0.053 ± 0.012 e
Non-RS	Cavendish	0.028 ± 0.001 a	0.028 ± 0.003 a	0.017 ± 0.001 c	0.020 ± 0.003 c	0.012 ± 0.002 d
	Plantain	0.024 ± 0.004 b	0.017 ± 0.002 c	0.017 ± 0.002 c	0.012 ± 0.001 d	0.011 ± 0.001 d
Total	Cavendish	0.209	0.142	0.092	0.077	0.039
	Plantain	0.277	0.218	0.157	0.092	0.064
RS/Total	Cavendish	0.866	0.803	0.815	0.740	0.692
	Plantain	0.913	0.922	0.892	0.870	0.828

Cavendish and Plantain fruit was harvested at the green maturity stage. After ethylene treatment, fruit were stored at 24 °C and 90% moisture, and sampled at 0, 2, 3, 4, and 5 d. Resistant starch (RS) and non-resistant starch (non-RS) contents were determined using a Resistant Starch Assay Kit. Data presented are the average ($kg\,kg^{-1}$ fresh weight) with SD of three replicates. Letters a, b, c, d, and e represent the significant differences with $P < 0.05$.