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Biochemical characterization of the flavedo of heat-treated Valencia orange during postharvest cold storage

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ABSTRACT

Heat treatment is a powerful and eco-friendly method to prevent *Penicillium* infection in citrus fruit during the postharvest. Several studies have been dedicated to investigate the general chemical changes that justify the immediate reaction responsible for the induced tolerance; but just how primary metabolism and enzymology are affected by heat treatment and along a prolonged cold storage is still unclear. In this work, the main enzymes of carbon metabolism of Valencia orange flavedo were analyzed during the postharvest period after a heat treatment (HT) of 48 h at 37 °C. Enzymatic activity measurements indicated that the NADPH producing enzymes glucose 6-phosphate dehydrogenase and non-phosphorylating glyceraldehyde 3-phosphate dehydrogenase have lower levels in HT fruit. In parallel, a higher synthesis of sucrose from organic acids was observed in HT epicarp. Sucrose-phosphate synthase would have an important role in sucrose accumulation. The pathway of carbon through glycolysis was affected by cold storage, independently of HT, in a way that it favors the ATP-dependent phosphofructokinase over the PPi-dependent homologous enzyme and the use of phosphoenolpyruvate (PEP) by PEP carboxylase instead of pyruvate kinase. Similarly, phenylpropanoid compounds did not show major changes in response to HT, although some of them showed a marked descent along the cold storage. Proteomic studies revealed alterations in the abundance of ascorbate peroxidase, two germin-like proteins and small HSPs, completing the description of the main metabolic changes in this tissue.

1. Introduction

Heat treatment (HT) is a frequently used conditioning method that help fruit and many other crop products withstand the postharvest period with low incidence of decay, chilling injury, insect attack and microbial infections (Ghasernnezhad et al., 2008; Lafuente et al., 2011; Lurie, 1998; Palou, 2013; Paull, 1990; Perotti et al., 2011; Sapitnitskaya et al., 2006; Schirra et al., 2011). It is a very low environmental impact method that can be applied alone or in combination with chemical treatments, reducing the amount to be applied and increasing its effectiveness (Palou, 2013). Ideally, HT must protect the fruit against decay while maintaining both internal and external quality unaltered (Palou, 2013). Different types of fruit respond differently to HT and, even within the same genus, considerable different responses may exist that rule out the possibility of using a single protocol in all cases (Lurie, 1998; Schirra et al., 2011).

The effects of HT on the general biochemistry of citrus fruit, which has been explored in the past, has received a boost in recent years by the use of modern techniques, mainly the application of the "omics" to unravel the molecular mechanisms subjacent to the response that HT elicits in the fruit tissues (Katz et al., 2007; Lara et al., 2009; Muccilli et al., 2009; Pan et al., 2009; Yan et al., 2006; Yun et al., 2013; Zhang et al., 2010).

In a recent paper, evidence was presented that HT bring out a number of responses in Valencia orange that are consistent with the improved resilience of the fruit during the postharvest period (Perotti et al., 2011). Most of the results and all the proteomic analysis were performed on the endocarp. This work completes the former study with a proteomic and biochemical examination of the flavedo in the same fruit. The flavedo of citrus fruit is a metabolically

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active tissue (much more than the endocarp at maturity) (Falcone Ferreyra et al., 2006) that, although does not constitute the edible part of the fruit, is essential to judge the quality of the fruit from a consumer's point of view. In citrus fruit, great differences exist in the response of this tissue to postharvest treatments and conditions. The study described here looks into the several metabolic changes introduced by postharvest condition in Valencia orange, probably the most consumed citrus fruit and one of the more resistant to the abiotic stress posed by postharvest management.

2. Materials and methods

2.1. Plant material and treatments

Assays were conducted with orange fruit [*C. sinensis* (L.) Osbeck] cv 'Valencia late' grown in the Estación Experimental Agropecuaria INTA, Concordia, Entre Ríos, Argentina (EEAC), harvested at full maturity in October/November 2007; and repeated with fruit grown during 2008. Immediately after harvest, fruit were manually selected for uniformity of color and size and divided into two groups of 50 each. The first group was used as control, remaining for 72 h at 20 °C. The second group received a heat treatment, which consisted of 48 h at 37 °C and 90% relative humidity followed by 24 h at 20 °C. This treatment usually is applied to oranges from the EEAC because its effectiveness in controlling *Penicillum digitatum* has been demonstrated previously (Cocco et al., 2008). Groups were labeled control (C) and heat treated (HT), respectively, and this couple of samples are called "fist pair". In turn, one subgroup of each category was stored at 4 °C for 60 d, simulating commercial conditions (C + 60 d and HT + 60 d, called "second pair"). Immediately after each treatment, the flavedo (epicarp) were frozen in liquid nitrogen and stored at −80 °C for further experiments.

2.2. Total protein extraction

Fruit tissue (approximately 0.5 g of epicarp) was powdered with liquid nitrogen in a mortar and then homogenized with ten volumes of extraction buffer (100 mM KPi pH 7.0, 1 mM PMSF). The final pH of the crude extracts thus obtained was near 7.0. The homogenates were centrifuged for 15 min at 4 °C in an Eppendorf microcentrifuge at maximum speed. The resulting supernatant was desalted in a Sephadex G-50 column previously equilibrated with five volumes of extraction buffer (Penefsky, 1977). The eluate was used as the source for enzyme activity measurements and inmunoblotting.

2.3. Protein quantification

Protein concentration was determined in crude extracts using a detergent-compatible formulation based on bicinchoninic acid (BCA) for colorimetric detection of total protein and bovine serum albumin as standard.

2.4. Activity assay

All enzymes were assayed at 30 °C in a Hitachi 150-20 (Hitachi Corp., Tokyo, Japan) spectrophotometer following the oxidation of NAD(P)H al 340 nm in a final volumen of 1 mL, except for fumarase, sucrose-phosphate synthase and succinate dehydrogenase (see below). The assays were optimized as described by Falcone Ferreyra et al. (2006). One basic experiment in which enzymatic activities were measured represents the mean of at least 3 determinations per fruit made in 3 fruit from each group. Each experiment was repeated at least twice. The reaction mixtures for each assay were as follows.

Glyceraldehyde-3-phosphate dehydrogenase (Ga3PDH, EC 1.2.1.12): 50 mM Tricine-NaOH, pH 8.5, 4 mM NAD, 2 mM fructose-1,6-bisphosphate (Fru-1,6-P_2), 10 mM arsenate and 1 U aldolase. Non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (npGa3PDH, EC 1.2.1.9): 50 mM Tricine-NaOH, pH 8.5, 0.4 mM NADP, 2 mM Fru-1,6-P_2 and 1 U aldolase. Malate dehydrogenase (MDH, EC 1.1.1.37): 50 mM imidazole, pH 6.9, 1 mM oxaloacetate and 0.15 mM NADH. NAD-malic enzyme (NAD-ME, EC 1.1.1.39): 50 mM HEPES-NaOH, pH 7.3, 2 mM NAD, 2 mM L-malate, 5 mM dithiothreitol (DTT), 75 μM CoA, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 2 U MDH. Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49): 100 mM Tris–HCl, pH 8, 0.2 mM NADP and 2 mM glucose-6-phosphate. Hexokinase (HK, EC 2.7.1.1): 30 mM HEPES-NaOH, pH 7.5, 2 mM $MgCl_2$, 0.6 mM EDTA, 9 mM KCl, 1 mM NAD, 2 mM glucose, 1 mM ATP, 1 U NAD-dependent glucose-6-phosphate dehydrogenase. ATP-dependent phosphofructokinase (ATP-PFK, EC 2.7.1.11): 50 mM Tris–HCl, pH 7.5, 0.15 mM NADH 4 mM fructose-6-phosphate (Fru-6-P), 0.5 mM ATP, 5 mM $MgCl_2$, 5% (w/v) polyethylene glycol (PEG), 1 mM dithiothreitol (DTT), 0.2 U aldolase and 0.1 U glycerophosphate dehydrogenase triose phosphate isomerase. Pyrophosphate-dependent phosphofructokinase (PPi-PFK, EC 2.7.1.90): 50 mM Tris–HCl, pH 7.5, 0.15 mM NADH, 4 mM Fru-6-P, 0.5 mM PPi, 5 mM $MgCl_2$, 5%(w/v) PEG, 1 mM DTT, 1 μM fructose-2,6-bisphosphate (Fru-2,6-P_2), 0.2 U aldolase and 0.1 U glycerophosphate dehydrogenase/triose phosphate isomerase. Fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11): 50 mM HEPES-NaOH, pH 7.5, 0.1 mM (Fru-1,6-P_2), 5 mM $MgCl_2$, 0.2 mM EGTA, 0.5 mM $NADP^+$, 2 U glucose-6-phosphate dehydrogenase and 1 U hexose phosphate isomerase. Phosphoenolpyruvate carboxykinase (PCK, EC 4.1.1.49): 50 mM HEPES-NaOH, pH 7.3, 2.5 mM $MgCl_2$, 2.5 $MnCl_2$, 10 mM $NaHCO_3$, 4 mM phosphoenolpyruvate (PEP), 0.15 mM NADH, 3 mM ADP and 2 U MDH. Activity was corrected for interference by PEPC activity by omitting ADP from the reaction mixture. Phosphoglucose isomerase (PGI, EC 5.3.1.9): 50 mM Tris–HCl, pH 8.1, 1 mM fructose-6-phosphate, 0.24 NADP and 2 U G6PDH. Phosphoglucomutase (PGM, EC 2.7.5.1): 20 mM imidazole, pH 7.85, 10 mM $MgCl_2$, 3 mM EDTA, 0.1 mM glucose-1,6-bisphosphate, 0.5 mM NADP and 0.8 U glucose-6-phosphate dehydrogenase. PEP carboxylase (PEPC, EC 4.1.1.31): 100 mM HEPES-NaOH, pH 8, 10% (v/v) glycerol, 5 mM $MgCl_2$, 10 mM $NaHCO_3$, 4 mM PEP, 0.15 mM NADH and 0.6 U MDH. NADP-malic enzyme (NADP-ME, EC 1.1.1.40): 50 mM Tris–HCl, pH 7.5, 0.5 mM NADP, 10 mM $MgCl_2$ and 10 mM L-malate. Pyruvate kinase (PK, EC 2.7.1.40): 25 mM HEPES-NaOH, pH 7.2, 20 mM KCl, 10 mM $MgCl_2$, 2 mM PEP, 0.15 mM NADH, 5% (w/v) PEG, 1 mM ADP and 0.4 U LDH. This enzymatic activity was corrected for interference by PEP-phosphatase activity by omitting ADP from the reaction mixture. Alanine aminotransferase (Ala-AT, EC 2.6.1.2): 100 mM Tris–HCl, pH 8.0, 25 mM alanine, 10 mM 2-ketoglutarate, 0.15 mM NADH and 0.2 U LDH. Glutamate oxaloacetate transaminase (GOT, EC 2.6.1.1): 80 mM Tris-Cl, pH 7.8, 20 mM L-aspartate, 15 mM 2-ketoglutarate, 0.15 mM NADH, 0.8 U LDH and 0.6 U MDH. Fumarase (EC 4.2.1.2): 100 mM KPi, pH 7.4 and 50 mM L-malate. This activity was measured following the production of fumarate at 240 nm. Neutral invertase (NI, EC 3.2.1.26): 200 mM HEPES-NaOH, pH 7.5, 200 mM sucrose. The mixture was incubated at 30 °C for different times and the progress of the reaction was followed detecting the amount of glucose produced by using glucose oxidase/peroxidase commercial kit (Wiener Lab, Rosario, Argentina). Acid invertase (AI, EC 3.2.1.26) was assayed under the conditions described above, although the reaction mixture contained 100 mM acetic acid/sodium acetate buffer, pH 5.0. In the case of AI, the aliquot was neutralized prior to glucose determination. Sucrose Synthase (SS, EC 2.4.1.13): 100 mM MES, pH 6.5, 3 mM $MgCl_2$, 0.5 mM EDTA, 5 mM β-mercaptoethanol, 50 mM sucrose, 0.02 mM glucose-1.6-bisphosphate, 0.5 mM NAD, 1 mM UDP, 1 mM PPi, 1 U PGM, 1 U G6PDH and 1 U UDP-glucose pyrophosphorylase. Sucrose-phosphate synthase (SPS, EC 2.4.1.14): the reaction mixture of 200 μl contained 100 mM