High Carbon dioxide Delays Postharvest Changes in RuBPCase and Polygalacturonase-related Protein in Cherimoya Peel

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Abstract. The peel of ‘Fino de Jete’ cherimoya (Annona cherimola Mill.) stored at 20 °C in air and in an atmosphere with high levels of CO2 was analyzed for changes in protein levels, color, chlorophyll content, and firmness. The accumulation of immunoreactive proteins was studied using Rubisco and polygalacturonase antibodies. During storage in air, cherimoya peel tissues exhibited a decrease in chlorophyll content and an immunodetected drop in the abundance of the large subunit of ribulose 1,5-biphosphate carboxylase (LSR). An immunodetected rise in polygalacturonase (PG)-related protein was quantified. High CO2 levels delayed the softening of cherimoya fruit, retarded the maximum accumulation of PG-related protein, and maintained LSR levels, greenness, and chlorophyll content of peel tissues.

Materials and Methods

Plant material. ‘Fino de Jete’ cherimoya (Annona cherimola) fruit of uniform maturity stage and size, and free from physical and pathological defects, were harvested in Almuñecar (Granada, Spain). One day after harvest, the fruit were transported overnight by road to our laboratory in Madrid; classified for uniformity of color, maturity, size, and weight; and divided into two lots. Twenty-five fruit were placed in a 20-L respiration chamber and ventilated either with a continuous flow of air or of 20% CO2 plus 20% O2. After 3 d under 20% CO2, the fruit were air-ventilated for 2 additional days. All cherimoyas were kept in the dark throughout the trials. Three individual fruit replicates per day were evaluated. The fruit was sampled after 1, 2, 3, and 4 d of air storage. Cherimoyas treated with CO2 were analyzed at the end of treatment (3 d) and 1 and 2 d after their transfer to air. Following treatments, each fruit was sliced in half to determine the precise stage of ripening. Peel tissue from each half-fruit was rapidly frozen in liquid nitrogen and stored at –80 °C. Changes in physiological stages were assessed by firmness and color measurements. The color measurements were made with a HunterLab tristimulus colorimeter (model D25A-9) calibrated with a white standard tile (X = 82.51; Y = 84.53; Z = 101.23) and measuring head geometry of 45/0 °C, 2° observer, CIE illuminant C, diameter specimen area 12.7 mm. L, a, and b values were assigned as the average for three representative samples, with three readings taken (on the pedicelar area) for each fruit. After obtaining three data sets, chroma C = (a2 + b2)1/2 was calculated. Firmness was measured in whole fruit at three places on the equator using an Instron (model 1140) testing machine fitted with a double-plate probe. Total chlorophyll in cherimoya peel was extracted with 80% (v/v) acetone and determined according to Strain et al. (1971).

Protein extraction. Frozen cherimoya tissue (10 g) was ground in a precooled coffee grinder and homogenized at 4 °C for 20 min temperature storage. This analysis was designed to determine the effect of high levels of CO2 on green peel tissues. The following have been characterized: 1) color modifications by measuring Hunter color scale values (L, a, and b), chlorophyll levels and ribulose 1,5-biphosphate carboxylase (RuBPCase) content and 2) texture modifications in terms of flesh firmness of the whole fruit and polygalacturonase (PG)-related protein content.

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in 30 mL of ice-cold extraction buffer [50 mM Tris-HCl (Tris (hydroxymethyl)-aminomethane) pH 7.4, 400 mM NaCl, 20 mM NaHCO₃, 20 mM MgSO₄, 10 mM sodium ethylene-diamino tetraacetic acid (EDTA), 20 mM 2-mercaptoethanol, 0.5 mM phenylmethyl-sulfonyl-fluoride (PMSF), 0.01 mM leupeptin, 10% (v/v) glycerol, and 1% (w/v) Triton X-100]. The homogenate was centrifuged at 20,000 × g for 20 min at 4 °C. The resulting supernatant was filtered through eight layers of cheesecloth. The soluble proteins were further purified using the phenol–ammonium acetate–methanol method (Montero et al., 1995). Protein concentration was measured by the Bradford method (1976). Bovine serum albumin dissolved in an adequate dilution of the SDS-PAGE modified sample buffer was used as a protein standard. Absorbances were read in a spectrophotometer (Perkin-Elmer UV/VIS Lambda-15).

Electrophoresis and immunoblotting. Protein samples were prepared for electrophoresis by adding four volumes of sample buffer consisting of 63 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 2% SDS, 5% (v/v) 2-mercaptoethanol, and 0.002% (w/v) bromophenol blue. Samples were heated at 95 °C for 4 min before electrophoresis and separated by SDS-PAGE on 14% polyacrylamide slab gels (15 × 11 cm and 1 mm thick) as described by Laemmli (1970).

Transfer of electrophoretically resolved proteins on SDS-PAGE gels onto nitrocellulose membranes of 0.22-µm pore size (Millipore, Bedford, Mass.) was performed essentially as described by Towbin et al (1979). The proteins were immunodetected with rabbit monoclonal antibodies against tomato LSR (dilution 1:20,000) or rabbit polyclonal antibodies against tomato PG (dilution 1:60,000). The membranes were incubated with antirabbit IgG horseradish peroxidase conjugate (Biorad) in TBS-T with 2% (w/v) milk and rinsed with TBS-T. Development with chemiluminescent substrates and exposure to X-ray films was performed following factory (Amersham) recommendations. Levels of antigen on the nitrocellulose filter were quantified using an image analyzer (BioImage and Visage, Millipore Corp., Ann Arbor, Mich.).

### Table 1. Color and total chlorophyll content in cherimoya peel during storage at 20 °C in the dark. Treatment A: fruit stored in air. Treatment B: fruit treated for 3 d in CO₂ (20%) atmosphere and transferred to air.

| Days in air | L | a | b | (a² + b²)²/² | Chlorophyll (µg·g⁻¹ fresh weight) |
|-------------|---|---|---|-------------|-----------------------------------|
| 0          | −8.21 ± 0.29 | 18.14 ± 0.54 | 19.91 | 106.17 ± 6.37 |
| 2          | −7.26 ± 1.22 | 17.25 ± 0.57 | 18.71 | 78.85 ± 2.00 |
| 3          | −6.71 ± 0.61 | 15.96 ± 0.60 | 17.31 | 72.45 ± 1.30 |
| 4          | −5.85 ± 0.53 | 14.40 ± 0.20 | 15.19 | 70.26 ± 1.05 |

| Days in air | L | a | b | (a² + b²)²/² | Chlorophyll (µg·g⁻¹ fresh weight) |
|-------------|---|---|---|-------------|-----------------------------------|
| 0          | −10.17 ± 0.56 | 18.73 ± 0.42 | 21.31 | 120.29 ± 2.10 |
| 1          | −7.66 ± 0.69 | 16.05 ± 0.93 | 17.78 | 115.84 ± 1.62 |
| 2          | −5.58 ± 0.31 | 14.85 ± 0.76 | 15.86 | 95.35 ± 0.75 |

Fruit after harvest.
Values indicate the means ± sd.
more tolerant to high levels of CO2 than white tissues (Lipton and Mackey, 1987). Furthermore, the chlorophyll content of green leaves and vegetables is maintained by high CO2 levels (Aharoni et al., 1989) and the rate of chlorophyll degradation in apple peel and cortex declined under low oxygen storage (Knee, 1980). However, relatively few investigators have rigorously examined whether the ripening-retarding effect of CO2 in fruit is due to decreased rates of degradation of the plastid components. As ribulose biphosphate carboxylase is the most abundant chloroplast protein, we selected this protein for analyzing the effect of CO2 on green tissues of cherimoya fruit.

Analysis of variance (ANOVA) was run to determine the significance of the data for significance level $P \leq 0.05$ using the Statgraphics program (STSC, Rockville, Md).

**Results and Discussion**

Changes in color and total chlorophyll content during storage in air and after high CO2 treatment are shown in Table 1. Hunter color scale values (L, a, and b) and chroma were used to measure changes in the color of cherimoya peel. During storage in air, cherimoya peel exhibited a slight loss of greenness and a yellow discoloration. The chroma value confirmed that the longer the storage period at 20 °C, the less chromatically green was the peel. During the first few days of storage at 20 °C, a significant decrease in total chlorophyll content was also recorded. The a value of cherimoyas treated with CO2 was significantly higher than for fruit stored for 3 d in air. However, the a and b values decreased significantly 2 d after the fruit was transferred to air. Carbon dioxide treatment likewise induced the retention of chlorophyll content. It is generally acknowledged that green tissues are
46-kD polypeptide showed the most notable changes during the ripening process. Even though the exact relationship of these polypeptides is still unclear, we believe, on the basis of the results for tomatoes (Tucker et al., 1980), that the 46-kD protein on SDS-PAGE gels could be the mature PG. PG protein was detected in cherimoya peel and pulp on day 0 of storage at 20 °C (lane 0 and 0*, Fig. 6A). It should be noted that the flesh firmness value of these fruit at the time was 73 N (Fig. 5), a value clearly lower than generally found in fruit immediately after harvest (100–110 N). The PG protein level increased during air storage. Dominguez-Puigjaner et al. (1992) found that a PG-related protein accumulated during the ripening process in bananas. Different tropical fruit, such as papaya (Paull and Chen, 1983) and mango (Abu-Sarra and Abu-Goukh, 1992), showed a good correlation between firmness and cell-wall-degrading enzymatic activity, particularly PG. The effect of CO2 treatment on PG protein accumulation is shown in Fig. 6B. This blotting revealed that cherimoya peel of fruit treated with CO2 had lower levels of PG protein than the peels

Fig. 6. Immunodetection of PG in cherimoya fruit stored in air with and without CO2 treatment. Total protein extracts were resolved by SDS-PAGE electrophoresis and transferred to nitrocellulose sheets. The membranes were probed and immunoassayed with PG antiserum at 1:60,000. (A) Cherimoya samples were stored in air at 20°C. Lane 0, peel of prestored fruit; lane 0*, pulp of prestored fruit; lanes 2, 3, and 4, peel of fruit stored in air for 2, 3, and 4 d, respectively; 10 µg of total protein were loaded in each lane. (B) Comparison of PG protein accumulation in peel of cherimoya fruit treated with and without CO2.

To determine whether the RuBPCase protein content was affected by high CO2 treatment, SDS-PAGE electrophoresis (Fig. 3a) and a subsequent immunoblotting assay were run on total protein extracts of cherimoya peel from fruit analyzed at the end of treatment with CO2 for 3 d, and after transfer to air for 1 and 2 d; the same LSR antiserum was used in both trials (Fig. 3b). In this case the protein content of fruit stored in air for 3 d was the control. Quantitative measurements (Fig. 4) showed that, at the end of CO2 treatment, the highest levels of RuBPCase protein accumulated in the peel. However, 1 d after transfer to air a sharp decrease in the abundance of RuBPCase protein was observed (Figs. 3b and 4). Although some authors have reported a relationship between such decreases and altered rates of RuBPCase subunit synthesis at the transcriptional and translational levels (Bate et al., 1991; Jiang et al., 1993), it is commonly assumed that they reflect enhanced rates of RuBPCase protein degradation (Brady, 1988). The decrease in RuBPCase protein content and the loss in chlorophyll level may indicate chloroplast dismantling in green tissues of cherimoya peel during storage in air at 20 °C, which is delayed by high CO2 treatment.

Cherimoya undergoes considerable softening during storage at 20 °C. A reduction of 50% of the initial value was observed after 2 d at 20 °C (Fig. 5). Continuous exposure to 20% CO2 for 3 d induced a greater retention of firmness (twice the value of fruit kept in air). However, after transfer to air, the fruit reached the same stage of softening as nontreated fruit at the end of the ripening period. Since this change in firmness may be an indication that cell-wall metabolism enzymes are affected by pretreatment with CO2, the PG levels in cherimoya peel were analyzed by blotting SDS-PAGE gel polypeptides extracted from the peel of cherimoyas ripened in air onto nitrocellulose filters and probing them with polyclonal antibodies raised against purified tomato PG protein (Fig. 6A). PG antiserum appeared to react with a number of PG-related proteins with a molecular weight of 55, 46, and 36 kD. The

Fig. 7. Histogram representing the quantification of changes in peel PG protein of cherimoya stored in air with and without CO2 pretreatment as described in the legend to Fig. 6. The immunoblots were quantified by densitometry and data are given as relative peak area. Similar patterns were obtained in three separate experiments.
of cherimoyas stored for 3 d in air. However, these levels increased when treated fruit were transferred to air (Figs. 6B and 7). Kanellis et al. (1991) reported that the rate of increase in avocado PG protein content was lower when stored under controlled atmospheric conditions. This cell wall hydrolase has been extensively studied during ripening and is believed to play a major role in the softening of tomatoes (Tucker and Grierson, 1982). However, the results obtained with transgenic tomato plants that express the antisense PG-gene imply that other cell-wall-degrading enzymes make an important contribution to softening, although the involvement of other hydrolytic proteins is likely to play an important role in this process.

We conclude that high levels of CO2 treatment delay the changes associated with storage of cherimoya fruit at ambient temperature, such as the decline in chlorophyll and RuBPCase activity in fruit stored in air or pretreated with CO2 seems to indicate that this protein may be associated with the softening of cherimoya fruit, although the involvement of other hydrolytic enzymes is likely to play an important role in this process.

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